MOLECULAR INVESTIGATION OF G-PROTEIN LINKED RECEPTORS IN THE CENTRAL NERVOUS SYSTEM

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DECLARATION

All of the investigations and procedures presented in this thesis were performed by the author unless otherwise stated.

This thesis was composed by the author.

ABSTRACT

The initial aim of this research was to discover and characterise new members of the G-protein linked receptor (GPLR) superfamily. There is an abundance of molecules which elicit a physiological response in the central nervous system (CNS) but have not yet had their mechanism elucidated. Because of recent work in this area there have been many new GPLRs discovered corresponding to many of these potential ligands and this area of research remains a rapidly expanding area of great interest in medical and physiological research.

Using sequence homology the receptors were grouped and degenerate primers were designed towards motifs specific to certain groups. These primers were then used to amplify sequences via the polymerase chain reaction (PCR) which could then be characterised.

The dopamine receptors are a subgroup of the GPLR superfamily containing five main members (D1 - D5) all related by sequence homology and their recognition of dopamine as the major ligand. They are of particular interest because they are the primary targets of drugs used in the treatment of psychomotor disorders (eg Parkinson's disease); however the reason for the range of the members with their different affinities for dopamine and different distributions within tissues still remains to be discovered.

This project aims to isolate members of this family by PCR and investigate their pharmacological properties in relation to each other.

ABBREVIATIONS

(β-)AR	(β-) adrenergic receptor
ATP	adenosine triphosphate
B_{max}	maximum amount of ligand that can bind to receptors in a membrane preparation
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
CNS	central nervous system
D_{2S}	short-form dopamine D_2 receptor
DEPC	diethylpyrocarbonate
DMF	dimethyl formamide
dopamine	3,4-dihydroxyphenylethylamine
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
EDTA	ethylenediamine tetraacetic acid
EtBr	ethidium bromide
EtOH	ethanol
fmol	femtomole
g	gram
GPLR	G-protein linked receptor
GTP	guanine triphosphate
^3H	tritium
IC_{50}	inhibitory concentration
IPTG	β-D-isopropyl-thiogalactopyranoside
kb	kilobase
K_D	ligand dissociation constant (mol l^{-1})
K_i	competing ligand dissociation constant (mol l^{-1})
l	litre
M	molar
ml	millilitre
µl	microlitre
NaOAc	sodium acetate
NK-1	neurokinin-1 (substance P)
nM	nanomolar
oligos	oligonucliotides
ORF	open reading frame
7-OH DPAT	7-hydroxy-N,N-di-*n*-propyl-2-aminotetralin

PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming units
PH/PTH	parathyroid hormone
pK_i	negative log K_i
pM	picomolar
RNA	ribonuleic acid
RNase	ribonuclease
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulphate
TACH	tachykinin
TE	Tris-EDTA buffer
TM	transmembrane domain
u	unit
VIP	vassoactive intestinal peptide
X-Gal	5-bromo-4-chloro-3-indolyl-β-galactopyranoside

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CHAPTER 1

INTRODUCTION

Research into novel G Protein Linked Receptors

G-protein linked receptors (GPLRs) are crucial components of intercellular communication and important targets for drug development. The work described in this thesis was aimed at:

a) the discovery of novel GPLRs, and

b) the investigation of the molecular biology of specific drug interactions on a particular subset of GPLRs, the dopamine D_2-like receptors

Therefore it is necessary to outline both the general properties and importance of GPLRs, and to describe the specific features of dopamine receptor biology and pharmacology.

Communication between cells and external factors is vital for the cells to respond in the appropriate way to allow development, co-ordinate growth, differentiation, perception, adaptation and ultimately survival of the organism. For this communication to occur there has to be a link between the exterior and interior of the cell to allow information to be relayed through the biological surface membrane which surrounds each cell. This transfer of information can be both by the physical movement of a substance in either direction, by diffusion for gases such as oxygen and carbon dioxide or via more complex systems, such as ion channels and surface receptors. Such highly specialised transmembrane signalling systems, along with a vast number of specific messenger molecules eliciting specific responses, enables the immense and complex functioning of the central nervous system (CNS).

1.11 Ion Channels

Ion channels are protein channels spanning membranes, which allow specific ion types (eg K^+ or Na^+) to pass through in a direction determined by their electrochemical gradient. Therefore, unlike diffusion, their action is controlled and the channels may be either open or closed depending upon triggers such as a change in voltage (voltage-sensitive channel), or a signal created by the binding of a ligand to an adjacent receptor molecule (chemically activated channel).

Ion channels are necessary because the lipid bilayer of the cell membrane has a high electrical resistance; therefore charged particles such as ions require a special passage which allows rapid transfer and give neurons the property of excitability (Barker *et al*, 1991).

1.12 Receptors

'Cell surface receptors are primary targets for pharmacological interference with physiological processes' (Burbach & Meijer, 1992); they are membrane spanning proteins which are components of a signal transduction system. The receptor receives extracellular signals, by the binding of a specific ligand, and relays the signal through the membrane (probably by a conformational change) to an intracellular component which effects a cascade of events, in response to the initial stimulus; therefore the receptor also amplifies and integrates extracellular signals (Dohlman *et al*, 1991).

Nearly all receptors and their subtypes are expressed in the brain and this is thought to provide the cells with a greater potential adaptive function for CNS information handling capabilities (Schofield *et al*, 1990). CNS receptors are situated mainly in the post and pre-synaptic membranes and can be classified into three main families according to the components of their signal transduction

mechanism, however their classification is becoming more difficult as the range increases with the discovery of new members. The main classes are:

1) receptors with an intrinsic ligand-gated ion channel (eg nicotinic acetylcholine receptors)
2) those containing an intracellular enzymatic domain (eg insulin receptors)
3) those coupled to a guanine nucleotide-binding regulatory protein (G-protein) which in turn triggers the appropriate second messenger system or ion channel (Burbach & Meijer, 1992).

This scientific study focuses on the latter group of G-protein linked receptors (GPLR). This is a fast expanding superfamily of receptors found as different populations in most tissues of most organisms from viruses and bacteria to humans. They are of great significance in the elucidation of CNS function and control.

1.2 Drug Development

GPLRs are involved in the transduction of many diverse extracellular signals including light, odorants, biogenic amines (products of cellular metabolism) and peptides. They consequently are of great interest from the view of drug development for major human disorders including those of the digestive system, heart and circulatory system, and of the central nervous system (CNS) (Dohlman *et al*, 1991).

Discovery of new receptor subtypes and specific ligand tests have become the basis of drug development. In the past few years this area has expanded greatly, especially on the molecular front. This opens up the possibility of great advances in the development of drugs for the associated physiological diseases. The increasing speed of discovery of new receptors and the increase in understanding

of their functions and interactions illustrate the impact of molecular research in this field. Technical advances have allowed the pharmacology, biochemistry and molecular genetics of the whole of mechanism of signal transduction and its associated effector processes to be studied.

It has been discovered that the involvement of GPLR in disease aetiology can be direct when sequence mutations cause the receptor to be constitutively active. An example of this is a somatic mutation in the thyrotropin receptor which causes constitutive stimulation of adenylyl cyclase and produces hyperfunctioning thyroid adenomas leading to hyperthyroidism. This occurs because the mutation is at a region of association with the G protein (G_S) and seems to eliminate the desensitisation mechanism. The receptors therefore are acting as proto-oncogenes producing excess cAMP which stimulates function and growth in the thyroid tissues (Parma *et al*, 1993).

Alternatively the receptor may not be the direct cause of illness but acts as a convenient point for drug intervention as with the treatment of Parkinson's Disease with L-Dopa.

Those GPLR found in the CNS are of particular interest in the study and treatment of neurological and psychiatric disorders such as Parkinsonism, Alzheimer's Disease and schizophrenia. The cloning of these receptors is an important advance in improving existing and developing new drug synthesis programs and is vital in discovering the extent of CNS involvement in the way the drugs have their action.

1.3 History Of G-Protein Linked Receptor Research

The field of receptor research has evolved from the early work of people such as Ahlquist (1948), who first divided the adrenergic receptors into pharmacological subtypes. During recent years great advances have been made, with the cloning of

increasing numbers of new receptors and additional subtypes of previously discovered receptors. Subtypes are defined as those members of a receptor family with significant structural and sequence homology but differences in the coupled effector pathway and ligand binding pharmacological profiles (Schofield *et al.* 1990). As new subtypes are detected, the number and size of subclasses in the GPLR superfamily increases, in some cases leading to new groups being defined, for example the 'VIP-like' class, (including the secretin, bombesin, and VIP (vassoactive intestinal peptide) receptors). This increase in number can cause problems with nomenclature and classification.

Now that the sequences of many members of the GPLR superfamily have been obtained, the DNA directed approach is the principal area of new advances. Cross-species screening has revealed equivalent receptor genes in different species and new related receptors are constantly being discovered using degenerate oligos for conserved regions in the transmembrane (TM) regions (Libert *et al,* 1989). Originally the technique of library screening was sufficient to uncover relatives however PCR has become increasingly important in uncovering those receptors present at very low levels in tissue (Burbach & Meijer, 1992).

Two receptors of the GPL type which have been of particular significance in the study of this superfamily, are rhodopsin and the β-adrenergic receptors (β–AR). Rhodopsin mediates conversion of light energy into a neurochemical signal (phototransduction). It is synthesised only in the retinal rod cells, at very high concentrations, which has facilitated its purification and subsequent characterisation. The bovine gene was cloned first in 1983 followed by the human gene the following year (Nathans & Hogness, 1984). Analysis of the sequence revealed seven stretches of hydrophobic amino acids representing possible membrane-spanning domains. This hypothesis was derived using the hydropathy analysis of Kyte and Dolittle (1982), which indicated a very similar profile to that

of bacteriorhodopsin - a non-G-protein linked membrane proton pump protein from *Halobacterium halobium.* This protein structure had previously been determined by image reconstruction from electron microscopy and found to contain seven α-helices spanning the lipid bilayer (Henderson *et al,* 1990). Proteolytic mapping of rhodopsin and β_2-adrenoceptor supports this topography (Ovinnikov, 1982) and although still the subject of controversy due to the lack of structural evidence for other GPLR this structure has become a characteristic of GPLRs (Hoflack *et al,* 1994), (Figure 1.3, p6a).

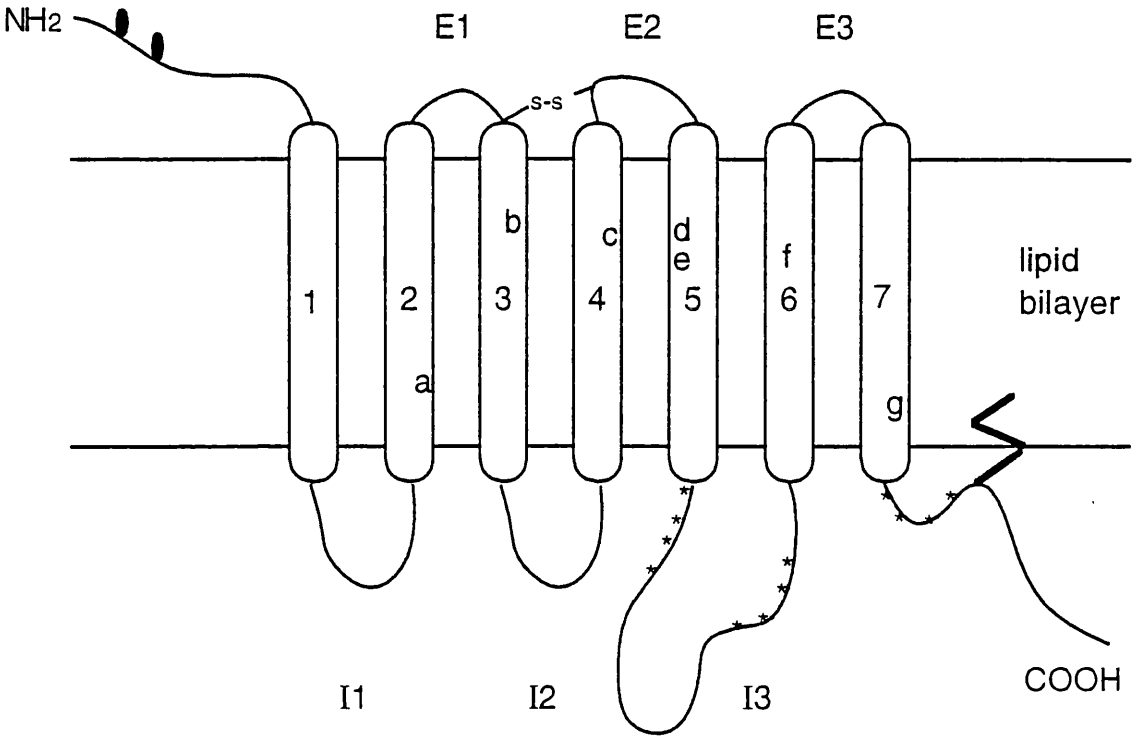
1.31 Adrenoceptors as Model Receptors

The adrenoceptors (AR) have been studied as the model system for the mechanisms of GPLRs since the first of their class, the mammalian β_2-adrenoceptor (β_2-AR), was cloned and found to be homologous to rhodopsin, relatively early on (Dixon *et al,* 1986). It has since been particularly useful for relating structural information to biological function.

The previous attempts to clone these receptors had encountered difficulty, due to the problem of purification. β-AR are only present at very low concentrations in most mammalian tissues (~0.001% mRNA) which has been a difficulty common in all GPLR studies (Lefkowitz *et al,* 1983). This has now been overcome by advanced techniques such as polymerase chain reaction (PCR).

Initially, the production of cAMP had been demonstrated to be controlled by the catecholamine neurotransmitters adrenaline and noradrenaline binding to receptors distinct from the effector enzyme adenylyl cyclase (Robison *et al,* 1967). A third component of the system was discovered when the requirement of guanine triphosphate (GTP) was observed for hormonal activation of adenylyl cyclase, in vitro (Rodbell *et al,* 1980) and subsequently an adenylyl cyclase-stimulatory, GTP-binding protein (G_s) was purified and cloned (Gilman, 1987). Prior to

Figure 1.3 Proposed membrane topography of G-protein linked receptors. Sites marked a-g indicate proposed interaction sites for ligand binding to B-adrenergic receptors (table 1.7a).



Key : a - asparagine 79
b - asparagine 113
c - serine 165
d - serine 204
e - serine 207
f - phenylalanine 290
g - tyrosine 326

● - glycosylation @ N6 & N15

> - palmytolation @ C341

* * * - regions involved in G-protein coupling

The putative disulphide bridge between cysteine residues on E1 and E2 is shown as are the glycosylation sites on the N-terminus (N6 & N15) and the palmytolation site on the C-terminus (C341)

cloning the receptors involved had already been identified and subdivided into α, β_1 and β_2 types, using ligand binding studies (Lands *et al,* 1967).

Today there are nine distinct cloned AR subtypes, six alpha and three beta, alike in sequence homology but pharmacologically discrete. Research has revealed a great deal about their signal transduction mechanisms which can be applied through structural homology to GPLR in general.

The β_1 and β_2 adrenoceptors were the first GPLR to have their signalling pathway elucidated (Kobilka *et al*; 1987; Frielle *et al,* 1987), from the involvement of the stimulatory G-protein intermediary, to the coupling to the second messenger/effector system adenylate cyclase which brings about a cascade of further events including cAMP dependant phosphorylation of many target proteins (Summers & McMartin, 1993).

1.4 G-Protein Interactions and Second Messenger Systems

Kobilka *et al* (1988) produced chimeras from α_2 & β_2 adrenoceptors to study receptor-G-protein interactions. The receptors respectively inhibit (via G_i) and stimulate (via G_s) adenylyl cyclase but are both activated by adrenaline. By studying the ligand binding and effector activating properties of these chimeras within cell systems, structural domains were deduced, determining the specificity of ligand binding and effector coupling.

The results showed that the 5th and 6th TM domains, the 3rd cytoplasmic loop and the C terminus contained specificity for the G_s coupling to the β_2 adrenoceptor, confirming that G-protein interaction with the receptor is complex and can not be isolated to one particular domain, preventing allocation of specific G-protein interactions by identification of specific receptor consensus sequences.

The present knowledge of the transmembrane signalling system indicates the following situation:

1) a receptor spanning the cell surface membrane
2) a G-protein situated on the inside surface of the membrane and able to interact with both the receptor and effector
3) an effector/second messenger system - eg adenylyl cyclase, phospholipase C, ion channels or other enzymes such as cGMP phosphodiesterase (as in the case of rhodopsin) (Dohlman *et al,* 1991).

All three peptides in the pathway are membrane associated. Binding of the extracellular agonist turns the intracellular face of the receptor into a catalyst that can dock with the G-protein and promote the exchange of cytoplasmic GTP for the GDP usually bound to the G-protein at rest. (Sternweis & Pang, 1990; Hille, 1992).

Following the discovery from adrenergic receptor research that control of cAMP production was due to the binding of extracellular catecholamines to a receptor discreet from adenylyl cyclase, it was found that receptor signal transduction was regulated by GTP (Rodbell *et al,* 1971). This initial work was performed on hormone receptors and was the first indication of the existence of G-proteins. Now it is recognised that the action of many stimuli including odorants, photons, cytokines, peptides, neurotransmitters and even sensory stimuli is mediated by receptor signal transduction pathway involving G-proteins.

Since the cloning of the first G protein (G_s), which stimulates the adenylyl cyclase system (Gilman, 1987), many forms of G-proteins have been discovered. They fall into several groups as classified by their α subunit:

a) G_s - stimulates adenylyl cyclase and regulates calcium channels

b) G_i and G_o - affect several secondary messenger systems including inhibition of adenylyl cyclase, stimulation of cGMP phosphodiesterase and regulation of ion channels

c) G_q - stimulates phospholipase C

Unstimulated, the G complex is a stable heterotrimer of three subunits (α,β & γ) which dissociates into two parts when GDP is replaced. The α-GTP moiety associates with the effector (eg adenylyl cyclase, phospholipase C) and influences second messenger production (eg cAMP, inositol phosphate (IP_3)). The $\beta\gamma$ moiety also acts as a signal for different effector subtypes, in the case of rhodopsin triggering an alternative primary effector for rhodopsin (Sternweis & Pang, 1990).

The point at which the receptor couples to the G protein is amplificatory and pleiotropic. Many G protein molecules of the same species are stimulated by the receptor (when bound to an agonist) and multiple systems are subsequently affected. However, different receptors have the capability to bind to several different species of G-protein in certain orders of selectivity depending upon their primary sequence, secondary structure and host cell environment (Watson & Arkinstall, 1994).

The α, β and γ subunits of G proteins are encoded by separate genes. To date there are at least 17 α, 4β and 6γ genes, with more products possibly produced by alternative splicing (Simon *et al,* 1991). The greatest diversity is found in the α subunit group, which is expected as this subunit performs the physical interaction with the receptor. This subunit also contains an integral GTPase activity and carries the GDP moiety associated when the G-protein is at rest.

The receptor, when stimulated by agonist binding, physically couples to the α subunit of the appropriate heterotrimeric G-protein. This triggers the exchange of bound GDP for cytoplasmic GTP raising the G-protein momentarily to an activated state. Whilst activated the α subunit dissociates from the receptor and from the β and γ subunits which remain as one unit, and these then regulate effector activities. The α subunit's innate GTPase activity hydrolyses the GTP, returning the subunit to the inactive state allowing reassociation with the βγ unit. Antagonist binding does not trigger the exchange of GDP to GTP and hence does not trigger signal transduction (Hille, 1992).

1.5 Receptor Topology

The problem in obtaining a 3D model of any of the GPLR superfamily is that due to their low abundance and membrane association. Manipulation is difficult and it has not been possible to generate ordered 2D and 3D structures from the proteins or to carry out X-ray crystallographic analysis. This however has been performed for bacteriorhodopsin (Henderson *et al,* 1990), an integral membrane protein which is a light-driven proton pump from the prokaryote *Halobacterium halobium.* Although not G-protein linked, this protein bears a functional resemblance to the mammalian membrane bound photoreceptor rhodopsin (they are both involved in photoisomerization of a covalently linked retinal chromophore) and a 3D folding pattern identical to that predicted for rhodopsin and other GPLR (Savarese & Fraser, 1992). Through further structural studies a topological model for bacteriorhodopsin was established, which could be applied to rhodopsin and in turn to GPLR in general (Ovchinnikov *et al,* 1979 & 1982).

Much of the current structural information has been deduced from rhodopsin and β-adrenergic receptors and applied to the many other members of the family using hydrophobicity profiles (Lentes *et al,* 1993) and primary sequence homology analysis (Dixon *et al,* 1986). However, there have been other modelling

approaches using amino acid side chain interaction energies (Roper *et al,* 1994) and computer generated 3D models (Hibert *et al,* 1991; Probst *et al,* 1992; Baldwin, 1993) using combined sequence and structural information. All methods result in the same general structural conclusions:

1) The general structure consists of 7 segments of 20-28 hydrophobic amino acids, which are predicted to form α-helical structures.
2) These α-helices are arranged through the lipid bilayer and linked by 3 extracellular and 3 intracellular loops of residues.
3) The α-helices are orientated so that polar residues, conserved residues and any residues known to be involved in ligand binding, are located towards the inner core of the model, so a 'binding-pocket' is formed.
4) The N terminus of the protein lies to the exterior of the cell membrane and the C terminus to the interior (cytoplasmic side).

However they differ in the finer details of helical arrangement (Donnelly *et al,* 1994).

It is important that work continues into verifying bacteriorhodopsin as a template for GPLR modelling as this is crucial in understanding ligand binding and rationalising drug design (Hoflack *et al,* 1994). Some controversy over the validity of bacteriorhodopsin appears to be regarding the differences in electron diffraction projection maps for bovine rhodopsin and bacteriorhodopsin (Hibert *et al,* 1993). This confirms that both proteins have seven transmembrane α-helices, but that they may not have the same topology. However the situation appears to be explained by differences in packaging of the helices and the angles with which they penetrate the membrane (Baldwin, 1993; Hoflack *et al,* 1994). Also it has been discovered that bacteriorhodopsin has different crystal forms depending on

the crystallisation conditions, which affects the protein packaging and may explain the differences in projection maps (Schertler *et al,* 1993).

A further general feature is the mechanism of signal transduction, elucidated by work on the β2-adrenoreceptor (O'Dowd *et al,* 1989; Summers & McMartin, 1993). Members of the GPLR superfamily have been shown to be analogous to these model receptors in both structure and function.

1.6 Evolution

The conservation of amino acid sequence and domain organisation suggests that these mechanistically related receptors have evolved by gene duplication from a common ancestral gene. The existence of so many genes for this information handling capacity indicates the biological importance of their function for evolutionary survival and species adaptation presumably by increasing the information handing capacities of tissues. This adaptive system offers the greatest flexibility for the vast heterogeneity of receptor subtypes (Schofield *et al,* 1990).

Many of the genes encoding GPLR have been mapped in both rodents, and more recently humans, and the results show that the loci are well dispersed throughout the genome. Interspecific backcross analysis was used and the data was used to predict human chromosomal locations based on linkage homologies identified between human and mouse. Even close family members are at distant loci, for example the dopamine receptors were mapped to human chromosomes 11 (D_2 & D_4), 3 (D_3), 4 (D_5) and 5 (D_1) (Wilkie *et al,* 1993).

It is known that basic cellular transmitters and their receptors developed very early phylogenetically (Walker & Holden-Dye, 1989). Subsequent evolution of many more receptor subtypes was required as the nervous system became more complex. To explore the relationship among the GPLR superfamily, a

phylogenetic tree has been constructed using nucleotide sequence information, which suggests that two of the major groups the opsin and catecholamine receptors diverged from a common ancestor over 1 billion years ago (Yokoyama *et al,* 1989) and the fact that these receptors are present in such a wide range of species including yeasts, indicates the importance of their function.

There are two mechanisms by which the superfamily could evolved to such a size and complexity. Duplication of existing genes appears to be likely (Onho, 1970) and is indicated by the relative preservation of intron location, which tend to be between TM domains. The D_2-like dopamine receptors are a good example of this, but there is also positional conservation to a lesser extent between this group and the tachykinins (Probst *et al,* 1992). The second mechanism is retroposition, as most of the receptor genes are intronless, indicating that they arose through reverse transcription of mRNA and incorporation into the genome (Brosius *et al,* 1991). However, convergent evolution is also evident as some receptors show no sequence relation to others (eg the secretin/VIP group).

Alternative splicing is also involved to a lesser extent in producing separate receptor isoforms. An example of this is the D_2 receptor gene which produces a splice variant lacking 29 amino acids of the third cytoplasmic loop (D_{2short} and D_{2long}, see below), (Grandy *et al,* 1989).

1.7 Associating Structure to Function

With respect to amino acid sequence the transmembrane domains have been found to be the most conserved regions and the remaining loops and termini more divergent. Together with the structural information available this has led to investigation of various receptor structure/function relationships (Attwood *et al,* 1991).

From a comparison of different areas of coding sequences it appears that genes from different receptor classes are subject to different selective pressures. For example the two groups adrenergic and muscarinic acetylcholinic receptors have some shared conservation especially in the transmembrane domain sequences, but also different patterns of conservation within their own groups involving the loop regions (Burbach & Meijer 1992). The same occurrence has been recorded for neuropeptide GPLR, when sub-grouped as secretin/VIP-like and neurokinin-like receptors.

For ease of comparison the amino acid sequences have generally been divided into domains E1-3 (the three extracellular loops), I1-3 (the three intracellular loops), TM1-7 (the seven transmembrane a-helices) and the C (carboxy, internal) and N (amino, external) termini. This overcomes the difficulties caused by differences in the length of receptor sequences, allowing for a more accurate alignment.

1.71 Transmembrane Domains

Even though these are the most conserved domains the degree of homology depends on how closely receptors are related. A study comparing the β_2-AR and muscarinic receptors revealed that the most conserved regions within these domains were those nearest to the cytoplasmic side of the membrane. This may be due to interactions with common cytoplasmic effectors, ie G proteins which have highly conserved structures. The extracellular sides are less conserved, and as the ligand interaction is likely here, this may possibly be due to the individual ligand binding profiles (Dohlman *et al*, 1988).

A great deal of work has been done by many groups on sequence conservation and this has identified a variety of conserved residues specific to different subclasses, which are possibly involved in ligand binding. (Strader *et al,* 1994; Probst *et al,* 1992) (Tables 1.7a & 1.7b).

Several proline residues are conserved in TM4, 5, 6 and 7 between many groups within the GPLR superfamily. These residues aren't commonly found in α helices as they cause kinks to the structure. It is expected that such conservation indicates an involvement in the tertiary structure required for functional activity. Brandl & Deber (1986) proposed that they may be responsible for conformational changes, triggered by ligand binding, whilst Applebury & Hargrave (1986) suggest that the bent helices may interlock to create a ligand accommodating pocket.

The transmembrane domains are demarcated by positively charged residues at the internal surface. This is common in membrane integrated proteins and may determine the correct orientation of the protein in the membrane and secure it there (O'Dowd *et al,* 1989).

1.72 Intra and Extracellular Loops

The loops of residues linking the transmembrane regions of the GPLRs have variable lengths between receptor types, however the functions they perform seem to be conserved. The extracellular loops appear not to contain residues directly associated with ligand binding, but rather have a conformational role. Most receptors contain conserved cysteine residues in the extracellular loops E1 & E2 which form a disulphide bond essential for the correct interaction of ligands (Dixon *et al,* 1987). (See '1.8 Structural Modifications')

On the intracellular side of the receptor all three loops, along with the C terminus, are responsible for the G-protein interaction. Charged residues have been identified in several receptors at the membrane proximal regions of loop I2 (in the highly conserved DRY sequence) and I3 which when mutated reduce dramatically G-protein coupling. These sites along with other residues in the C terminus and TM2 and 6 permit the complex association to the G-protein (Probst *et al,* 1992).

Putative phosphorylation sites have also been discovered on I3, which are thought to be utilised in the process of desensitisation (O'Dowd *et al,* 1989).

1.73 Terminal Regions

There appears to be no general functional role for the amino terminus as it lacks homology in sequence of length. It has been suggested that it may be an uncleaved signal sequence (Singer *et al,* 1987) and may protect this end form proteolytic attack due to its possession of putative glycosylation sites.

The C terminus is thought to be involved in modulation of receptor sensitivity, in response to the intensity or frequency of stimulation. This is known as desensitisation and is a negative feedback regulation of receptor function involving a rapid reduction in response to continuation of the same stimulus, displayed by all characterised GPLRs (Dohlman *et al,* 1991). There are two forms of desensitisation. The homologous mechanism involves a single receptor molecule which becomes modified by phosphorylation at the serine and threonine sites on the C terminus (Thompson & Findlay, 1984) and the binding of inhibitory proteins to decrease activity. In extreme cases of prolonged agonist exposure the receptor may be 'down-regulated' where it is endocytosed from the membrane and destroyed. Heterologous desensitisation is thought to be a more general mechanism, initiated by the over stimulated second messenger (eg cAMP initiates desensitisation of adenylyl cyclase systems), (Perkins, 1991). The result of both mechanisms is attenuation of the G protein coupling stage of the pathway and mutational work on the TM7 adjacent region of the C terminus indicates it as an interaction site for the G protein (Ostrowski *et al,* 1992).

A significant number of serine and threonine residues in the C terminus of most GPLRs are substrates for specific phosphorylases, the protein kinases PKC and PKA, which act when the receptor is stimulated (Benovic *et al,* 1986). Mutants

for these residues have been shown to eliminate desensitisation but there is still argument against the theory as the pattern of conservation in this region is poor. (O'Dowd *et al,* 1989; Dohlman *et al,* 1987)

1.74 Chimeric Experiments and Ligand Binding

This approach has become more popular for the study of these functionally complex molecules, as conclusions can be drawn from qualitative changes in receptor function such as acquisition of new functions that can be correlated with specific protein sequences. The mutagenesis approach provides more limited information as the end result is loss of function however it can be used to pin-point the involvement of an area as small as one residue. Substitution mutagenesis also has be carefully evaluated as the result depends greatly on the nature of the replacement residue.

The structure receptors when modelled on bacteriorhodopsin appear to form a hydrophobic 'pocket' which would allow ligand binding (Baldwin, 1993). Ligand interaction sites occuring within TM regions of receptors provides more evidence for a binding 'pocket'. These conserved residues, when mutated, affect ligand binding and signal transduction (see Table 1.7a, p17a).

Although certain receptors are used as models when investigating certain aspects such as ligand binding it should be noted that this does not always give a realistic view not least because of the range of different ligands involved (Neve & Wiens, 1995). This is particularly obvious in the case of peptide receptors.

The proposed models for ligand binding in different classes of GPLR all take into account charged and aromatic residues within the 'pocket' and propose an interaction of ligand to receptor which could form a network of aromatic-aromatic

Table 1.7a Key sites for ligand interaction with biogenic amine binding GPLRs

Receptor	TM	Residue	Structure	Function
Rhodopsin	TM3	Glu113	side-chain	counterion for Schiff base
	TM7	Lys296	E-amino group side-chain	Schiff base formation with rhodopsin
β-adrenoceptor [a]	TM2	Asp79[b]		agonist efficacy, transduction of signal
	TM3	Asp113[c]	side-chain	counterion for ligand binding
	TM4	Ser165	OH side-chain	ligand binding site[d]
	TM5	Ser204[e] Ser204	OH side-chain	H-bond formation for agonists and signal transduction
	TM6	Phe290[e]		stabilizes aromatic ring of catecholamine ligands
	TM7	Asn312	side-chain	phenoxypropanolamine antagonist binding site [f]
	TM4 & TM5		binding pocket	multiple residue interactions determining agonist specificity [g]
D_2 dopamine	TM2	Asp80		ligand binding and signal transduction[1]
	TM3	Asp114		ligand binding site
	TM5	Ser194 Ser197		H-bond formation for agonists and signal transduction
	TM6	Leu387 Phe389 Phe390		conformational change for signal transduction
	TM7	Phe411		signal transduction

[Reviewed in Strader *et al* 1994]

a β-adrenoceptor is a model receptor for all biogenic amine GPLRs and members of the GPLR family in general

b a corresponding aspartic acid residue occurs in all biogenic amine receptors and has been shown in several to have the same function (eg α-AR, muscarinic, histamine)

c most GPLRs have this highly conserved residue which has been shown to have the same function in many (eg α-AR, angiotensin, $5HT_2$, NK1, D_2)

d this residue is hypothesised to have this function as it mutation results in processing error and non-expression

e these residues are conserved in all catecholamine receptor which suggests co-evolution with the ligands

f conserved in biogenic amine receptors which bind phenoxypropanolamine antagonists with high affinity

g region determined by chimeric work - no single residue determined specificity

1 Neve et al, 1991

and hydrogen bonding interactions possibly leading to the proposed conformational change (Trumpp-Kallmeyer *et al* 1994).

Work on the tachykinin receptors (neuropeptides) has discovered that binding for peptide receptors appears to be much more complex.

1.75 Peptide Receptors

The size of ligands for this group of receptors is generally much larger than for the biogenic amines. Some peptides can be up to 40 residues in size (eg glucagon) and therefore modelling suggests that binding can not be supported by TM interactions alone (Strader *et al*, 1994). Mutagenic and chimeric studies have revealed that extracellular regions are involved, including the N terminus (Regoli, *et al* 1989) and all three extracellular loops (Fong *et al*, 1992). Research has been unable to pinpoint interaction sites to individual residues in many areas. The discovery that the neurokinin peptides occur as different conformations when in solution has lead to the hypothesis that selectivity may be determined by the the degree of conformational compatability between each peptide and the binding pocket of the receptor (Savian *et al*, 1992). Some individual residues involved have been discovered both in the extracellular domains and in the TM regions. However the situation is further complicated by the seperate but overlapping binding sites for peptide agonists and non-peptide antagonists. The peptide agonists have essential residues for binding in TM2, TM7 and E1, 2 & 3 whilst antagonist binding sites occur in TM4, 5 & 6 (Table 1.7b, p18a).

1.8 Structural modifications

There are several biochemical modifications which are important in defining and controlling receptor function:

Table 1.7b Key sites for ligand interaction with peptide binding GPLRs based on NK1 receptor work

Receptor	Residue/Region		Function
NK1[1] (substance P)	FXGLM-NH_2 N-terminus		required for agonist activity in all tachykinin receptors
	Asn23 Gln24 Phe25	E1	required for high affinity endogenous agonist binding [2] (no individual residues show this effect)
	Asn96 His108	E2	
	Asn85 [3] Asn89 Tyr92	TM2	agonist binding via H-bonds
	Tyr287	TM7	agonist & antagonist binding
	Glu78 [4] Tyr205	TM2 TM5	agonist binding, essential for receptor activation
	Val116 Ile290	TM3 TM7	responsible for conformation of non-peptide antagonist binding pocket
	Gln165 His197 His265 Tyr287	TM4 TM5 TM6 TM7	binding sites for non-peptide antagonists
	Exterior ends of TM5 & TM6		important for non-peptide antagonist binding

[Reviewed in Strader *et al* 1994]

1 NK1 (substance P receptor) is a model receptor for all tachykinin and most other peptide GPLRs (eg CCK and angiotensin)

2 this indicates that the situation for peptide receptors is more complex than for biogenic amine receptors with some regions having functions which can not be assigned to individual residues

3 conserved in NK1, 2 & 3 receptors

4 conserved as glutamic acid/aspartic acid and tyrosine/phenylalanine in many GPLRs

1.81 Glycosylation

G-protein linked receptors contain putative glycosylation sites usually in the N terminal region but they may also be present in the carboxy terminus. These sites are consensus sequences containing asparagine residues which may become glycosylated. Work on the β_2-adrenergic receptor showed that endoglycosidase treatment decreased its molecular weight from 65kDa to the expected 49kDa confirming that the protein was glycosylated (Rands *et al,* 1990). It was also shown that of the 4 sites in hamster β_2-AR the 2 in the N-terminus were utilised and glycosylated whilst the 2 in the C-terminus were not (Dohlman *et al,* 1987[2]). Removal of glycosylation does not affect ligand binding of function of receptors but may be involved in determining the distribution of the receptor, or its level of expression (Saverese & Fraser, 1992). However disruption of glycosylation can be detrimental in some cases. Retinitis pigmentosa is a retinal degenerative disease caused by a mutation causing destruction of a glycosylation site in rhodopsin (Sung *et al,* 1991).

1.82 Palmitoylation

Most GPLR have one or more cysteine residue in the C-terminal region. The single residue present in β2-AR has been show to covalently bind palmitate, which may insert into the lipid bilayer forming a small fourth cytoplasmic loop. Site directed mutagenesis of this cysteine residue interferes with coupling of the receptor to its associated G-protein (Gs), (O'Dowd *et al,* 1989[2]).

1.83 Disulphide Bridges

Yet another conserved feature of GPLR is the presence of two further cysteine residues at conserved sites on the first and second extracellular loops (E1 & E2). In rhodopsin it has been biochemically demonstrated that these form a disulphide bond ('bridge') which is necessary for arranging the receptor in the correct 3D

conformation (Karnik & Khorana, 1990). Dixon (1987) also demonstrated that ligand interactions in β_2-AR are disturbed by mutagenesis of these residues.

1.84 Phosphorylation

As mentioned previously GPLR can be negatively controlled by phosphorylation in the process of desensitisation, where G-protein coupling is disrupted. The phosphorylation occurs as consensus sites rich in serine and threonine which are targets for protein kinases such as PKC and PKA. These sites have been found on all of the three cytoplasmic loops as well as the C terminus which may point to multiple interaction sites for G-protein coupling.

1.9 Aims of Research

The superfamily of GPLRs is increasing in size rapidly as new members and sub-groups are discovered. They are being uncovered via various pathways - homology to existing, cloned GPLR is being taken advantage of, with cDNA library screening (Van Tol *et al*, 1991) and more extensively PCR is being used to discover further members of existing sub-groups (Libert *et al*, 1989).

In addition, receptor pharmacology has been used to uncover totally new sub-types of GPLR, which do not appear to be related to other groups by sequence homology but are transmembrane receptors baring all of the topological characteristics of GPLR. An example of this is the secretin receptor (Ishihara *et al*, 1991) which was isolated by direct expression cloning.

This project aims to discover new members of the neuropeptide receptor sub-family using the property of sequence homology to existing receptors. Degenerate primer PCR will be used. This approach has previously been used successfully by several groups to extend receptor sub-groups, for example

Zastawny *et al*, (1994) discovered further members of the opioid sub-group in this way.

A selection of published peptide sequences for known neuropeptide receptor sequences will be taken and sub-divided by peptide sequence homology, then the degenerate primer sequences created from the consensus' of these sub-groups. This will allow design of primers with a limited degeneracy, retaining a certain degree of specificity for the sub-group.

If this approach is successful and new members are discovered these will be cloned and further characterised both molecularly and pharmacologically.

A particular area of interest for the discovery of new receptors are the ligands they bind and the binding process, which is important for the understanding of physiological processes and the development of drugs to treat disorders involving GPLRs.

An area that particularly interests me is the selectivity of binding which a receptor has for a specific drug, and the difference in selectivity between receptors which differ very little in peptide sequence, such as the tachykinin receptors. As all of the evidence indicates that the receptors have the same intrinsic topological structure, therefore indicating that the alteration in selectivity must be due to the residue differences either directly or via a conformational change.

To investigate this, it is hoped that any new receptor cloned will have high enough homology to an existing member of it's sub-group (from which the degenerate primers towards it were designed) to create functional chimeric molecules from the pair. Chimeras for different regions of the receptors could be expressed and tested pharmacologically for binding specificity, in comparison with the pharmacological

profiles of the uncombined receptors, and in this way a binding profile for the new receptor could be built up, along with information about the regions important for its ligand interaction and specificity.

It is hoped that the chimeras will be created using a PCR method involving recombinant primers along with both receptors as templates. This technique has successfully been used by England *et al* (1991) to produce m1 muscarinic/D_2 dopamine receptor chimeras.

SECTION A

CHAPTER 2

SEQUENCE ANALYSIS

2.0 Sequence Analysis Of G-Protein Linked Receptors

In order to design degenerate PCR primers, with the aim of amplifying novel neuropeptide receptors, it was necessary to analyse a selection of neuropeptide receptor amino acid sequences to see if they could be further sub-grouped according to peptide sequence homology. The peptide sequence was used, as residue differences and similarities are more obvious and also the degeneracy can be designed with mouse codon bias (as the PCR template is mouse cDNA).

The sequences were randomly selected from those published on the GenEMBL database at that time (http://www2.ncbi.nlm.nih.gov/genbank). Eighteen sequences in total were chosen with the proviso that they were all neuropeptide binding GPLRs and that they were derived from rodents if possible, as the template for PCR was prepared from mouse brain tissue. Unfortunately, at this time, it was impossible to obtain the whole range of receptors from the mouse however, the selection was narrowed to human, and rodent sequences, which have reasonable homology.

Table 2.11 Neuropeptide receptors analysed for sequence homology

Receptor	Species	Accession N°
thyrotropin releasing hormone	mouse	M59811
bombesin	mouse	M61000
NK-1	mouse	X62934
somatostatin	mouse	M91000
neurotensin	rat	P20789
δ-opioid	rat	L07271
parathyroid hormone	rat	M77184
secretin	rat	X59132
NK-2	rat	M31838
bradykinin	rat	X69681
cholecystokinin	rat	X01032
endothelinB	human	M74921
calcitonin	human	L00587
angiotensin	human	M93394
NK-3	human	M89473
neuropeptide Y	human	M84755
vasoactive intestinal peptide	human	L13288
N-formyl peptide	human	M60627

2.1 Classification of Receptors

The advent of computer software to analyse sequence data has proved revolutionary in molecular genetics as now huge amounts of data can be compared and regions of sequence conservation which may be important to function or structure can be discovered and subsequently examined experimentally. Also established motif patterns (eg promoters, zinc fingers etc.) can be recognised allowing ease of elucidation of the function of a newly discovered gene or protein.

Analysis of this type has helped to elucidate the molecular structure of the GPLRs (G-protein linked receptors) and has been important in determining the relationship between different members of the superfamily, establishing their evolutionary and functional relationships.

The University of Wisconsin Genetic Computer Group Software package (GCG) contains many useful molecular analysis programs. Some of these were used to create multiple sequence alignments of various neuropeptide receptor amino acid sequences. The peptide sequences were chosen as these allow a more clear comparison of the differences or similarities between receptors at a structural level, as this affects ligand interactions and receptors are usually classified according to their ligand binding character.

At present a certain group of GPLRs may be allocated into different subset depending on which properties of the receptor are considered. For example this may be its major ligand type (eg catecholamines, peptides, hormones etc), its physiological effects or tissue specificity, conservation of structural features or sequence motifs or by sequence homology, leading to confusion and overlap between subgroups of GPLRs.

Much of the early confusion arose because of the naming system, whereby one receptor could have two or even three names, depending upon the number of groups which cloned it. For example the neurokinin-1 receptor (NK-1R) was known until relatively recently as the substance P or neurokinin A receptor and the bombesin receptor was also known as the gastrin releasing hormone receptor (Watson & Arkinstall, 1994).

The situation has now been clarified by the intervention of the International Union of Pharmacology (IUPHAR), a committee producing an annual supplement in the journal Trends in Pharmacology, reviewing and classifying receptors according to a standard nomenclature.

The analysis in this study compares the entire peptide sequence of several neuropeptide receptors allowing allocation of receptors into subsets by sequence homology. This type of analysis is useful from a molecular point of view and is important in the study of evolutionary relationships. Several homology studies on GPLRs have only analysed the transmembrane (TM) regions of the receptor sequence in order to eliminate the variation in length between receptors. This variation can occur both in the terminal and loop regions of the proteins and therefore could cause problems when trying to align two receptor sequences of different lengths. The multiple sequence alignment program on GCG, 'PILEUP' allows for this variation, producing the overall best alignment by introducing gaps into shorter sequences at positions calculated to give the least disruption and shift the sequence to the best alignment with its longer counterpart; it also allows for varying termini lengths.

2.2 Computer Analysis

The peptide sequences of 18 neuropeptide receptors were obtained from the GenEMBL database and converted into open-reading frame peptide sequences

using TRANSLATE (Wisconsin GCG). These were entered into the 'PILEUP' program which produced a multiple sequence alignment using the progressive, pairwise alignment method of Feng and Doolittle (1990). These sequences were chosen as a selection of neuropeptide receptor sequences on the GenEMBL database at the time of analysis. Translation to amino acid sequence was necessary in some cases, as many were not present on the SwissPROT database.

The method 'clusters' most alike sequences to create a dendrogram which is not phylogenetic but determines the likeness of sequences to each other and directs the order in which sequences are aligned. A total pairwise alignment of the sequences is then produced starting with the two sequences with highest homology, proceeding to next most homologous sequence and so on until all sequences have been aligned. The final output alignment therefore displays a multiple sequence alignment with the most similar sequences adjacent to each other.

The total alignment of all of the receptor sequences studied allowed the sequences to be subgrouped according to their 'pairwise similarity score' and aligned into a multiple sequence file. The program 'PRETTY' could then used to derive a consensus from the alignment and display it below.

2.3 Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a powerful technique which amplifies a specific sequence from an initial low concentration in a complex mixture (Mullis *et al*, 1987). Its success relies upon the careful design of experimental primers and calculation amplification conditions.

2.31 Experimental Design

The primers are the most crucial part of the technique as they are the key to detecting and amplifying the target sequence. It is important that each primer in the pair is designed with equal care as amplification will only occur when these two independent probes hybridise specifically and close enough to specify a target sequence. There are some general rules on the design of primer pairs:

- primers should contain at least 15 bases of homology
- the T_ms of the primer pair should be balanced and ideally lie between 55-80°C
- the last three 3' bases of the primers should be totally homologous to the target sequence
- the primers should have little or no complementarity to each other at the 3' ends to avoid primer-dimer formation (Innis & Gelfand, 1990)

Once designed the primer sequences were checked to confirm homology to the target sequences on the correct coding strand using the GCG program 'BESTFIT'. Also the primer sequences were checked to avoid homology between opposite members of each primer pair or palindromic/secondary structures within the primers. Secondary structures were checked for using the 'FOLD' program on GCG (Eberhardt, 1992).

2.311 Degenerate PCR

The situation becomes more complicated with the use of degenerate primers as the reaction must be specific enough to amplify related sequences without being indiscriminate. When an oligonucleotide is designed from an amino acid sequence, the occurrence of several codons for each residue means that a mixture of nucleotide sequences of the same length are created. These primers are useful for detecting the gene of a novel peptide or to amplify new genes, related to those from which the primers are designed.

Degenerate PCR has been successfully used in several cases to discover new members of the GPLR family. A typical example this is shown by the discovery of a somatostatin receptor by O'Carroll *et al* (1992) using degenerate PCR primers based on conserved regions of other related receptors, on a rat pituitary cDNA library. The method relies on the structural conservation of the different receptors in the GPLR family. The seven transmembrane domains offer regions of sequence conservation which can be aligned to provide an amino acid motif particular to a subgroup of receptors; these regions can then be used for degenerate primer design specifically towards that group. Therefore primer pairs are commonly designed towards the most highly conserved regions at an easily amplified interval from each other, eg TM domains 3 & 7 or 2 & 6 (O'Carroll *et al*, 1992). Amplified products can then be sequenced and libraries probed using any putative novel receptor sequences, to isolate full length cDNAs.

As primer design is more complicated than in non-degenerate PCR, there are further steps which may be taken to reduce primer complexity and hence increase specificity. In this experiment the specificity was increased by designing primers with a low degeneracy (below 516-fold). This was done by avoiding peptide regions containing amino acids with high codon usage and by using mammalian codon bias (Maruyama *et al*, 1986). Also the substitution of the nucleotide inosine

(which will pair with any base) at points where there is four-fold base redundancy, was used to reduce overall degeneracy (Compton, 1990). Degeneracy specifically at the 3' end of the primers was avoided where possible, as a mismatch in this region can impede extension of the primer (McPherson *et al*, 1990)

The primers were designed towards TM3 & TM7 because these regions have successfully been used by other groups and produce an easily amplifiable fragment (≈500-600bp), which is long enough to contain many of the major ligand binding residues (Figure 1.3, p6a) and the I3 loop which is likely to contain any variation in G-protein linkage residues.

The 3' region of homology was designed to be >15 nucleotides so that the primer could accurately anneal but a Kpn1 restriction site could be incorporated into a non-homologous 5' end. Using the program 'MAP' on GCG, it was shown that Kpn1 did not cut within the template receptors but will cut near the ends of DNA fragments.

2.32 Technical Considerations

The other major element in PCR is the template. It is possible to use either a preconstructed library (usually cDNA) or uncloned single stranded cDNA. The important fact is to be sure that the template complex contains the target sequence. In this experiment cortex and striatum RNAs were extracted and reverse transcribed. It was know that within brain regions all of the receptors used to design the primers were represented and there was evidence of further subtypes (Watson & Arkinstall, 1994).

2.33 Reaction Composition

Once the primers were made it was important to optimise the conditions for amplification. There are certain guidelines, but optimisation of a new set of primers requires much trial and error. Factors to consider include mainly primer & Mg^{2+} concentration and annealing temperatures:

- primer concentration should be 100-200pM ; too high a concentration leads to mispriming
- the annealing temperature should be around 5-10°C below the T_m (melting temperature) of the primer pair
- magnesium ion concentration should be between 0.5-2.5mM; concentration can affect primer annealing, primer-dimer formation and enzyme activity and fidelity
- deoxynucleotide triphosphates have to be at equal concentrations between 20-200μM; for ease a 2mM stock mix of all four nucleotide was used, care was taken to avoid multiple freeze-thaws as this can reduce efficiency.
- *Taq* concentration should be between 1-2.5 units/100μl reaction; too high a concentration causes accumulation of non-specific products

2.34 Amplification

There are three main steps:

a) Denaturation

Heating the template DNA to around 95°C for about 30 seconds is usually sufficient to denature duplexes to single strands ready for the next primer annealing step, any longer will lead to loss of enzyme activity. A 'hot-start' (94-96°C for 1-2mins) was incorporated into to the reaction prior to the first cycle. The *Taq* enzyme is added when the high temperature is reached in order to prevent extension of early non-specific primer hybridisations which could seriously reduce specificity.

b) Annealing

Conditions required for annealing of primers to the template depend upon the nature of the primer and it advisable to optimise the reaction for a particular pair of primers by trying different annealing temperatures, and varying Mg^{2+}, primer and enzyme concentrations (Wages & Fowler, 1993). A guide annealing temperature is 5°C below the T_m of the primers. At temperatures between 55 & 72°C with average primer concentrations (200pM) annealing should only require a few seconds (Innis & Gelfand, 1990). Increasing the annealing temperature increases the stringency and therefore the specificity of the reaction.

c) Extension

This is dependant upon the length and concentration of the target sequence. The usual temperature is 72°C as this has been shown to give the most efficient primer extension (Innis *et al*, 1988). One minute at this temperature is sufficient for a distance of up to 2 kb, but longer times are usually used to allow for low template concentrations.

Cycle number is also important. After a certain number of cycles (specific to the reaction) the exponential reaction stops and the amount of product plateaus. The problem with continued cycling after this point is that the level of non-specific products continues to increase (Wages & Fowler, 1993). It is suggested that 25-40 cycles should be used depending upon initial target molecule concentration, more than 40 cycles should be avoided for the above reason and also because even a single copy template should be amplified in 40 cycles.

RESULTS

2.4 Sequence Analysis

From the analysis of the amino acid sequences the neuropeptide receptors were assigned to groups according to a homology value determined using 'PILEUP'. This is a score of homology between each pair of receptors in every combination, with higher scores indicating greater homology up to a value of 1.0 which shows total homology.

Tables 2.41 to 2.45 (p34 & 35) show the sub-grouping according to peptide sequence analysis at TM domains 1-3 & 6-7. Domains 4 and 5 show much lower levels of homology and therefore have been omitted as it is difficult to define groups from these regions. Table 2.46 (p35) shows the sub grouping according to entire length sequence comparisons.

The sub grouping within the tables is determined by the homology of each peptide sequence for each other sequence, as derived by the 'PILEUP' application (Wisconsin GCG) and shown in Table 2.40, p32a.

The results of grouping at different TM domains (Tables 2.41 - 2.45, p34 - 35) show that the sub-group which a receptor falls into differs, depending upon which region is being examined. In some studies receptors have been assigned to sub-groups according to their homology only at TM regions, however, these results show that in this case it is more reasonable to assign grouping by sequence analysis of the entire reading frame so that the ambiguity of grouping by TM region sequences is eliminated.

In Table 2.46, as expected some established groups appear such as the tachykinins (group ii) and the 'secretin-like' receptors (group i). However there are some

Table 2.40

Quality ratios for all possible pairwise amino acid sequence alignments - derived using 'PILEUP' GCG - (chapter 3)

	sec	cal	pth	nk1	nk2	nk3	nt	npY	trh	bom	cck	end	ang	brad	Nfp	∂-op	soma	vip
sec																		
cal	0.48																	
pth	0.59	0.46																
nk1	0.34	0.32	0.33															
nk2	0.32	0.28	0.32	0.73														
nk3	0.31	0.31	0.35	0.84	0.66													
nt	0.34	0.33	0.34	0.4	0.42	0.42												
npY	0.30	0.32	0.30	0.41	0.38	0.38	0.42											
trh	0.30	0.31	0.30	0.45	0.38	0.39	0.45	0.35										
bom	0.33	0.32	0.31	0.4	0.39	0.4	0.43	0.37	0.4									
cck	0.30	0.33	0.32	0.4	0.44	0.45	0.44	0.36	0.43	0.44								
end	0.34	0.31	0.34	0.36	0.4	0.34	0.44	0.35	0.41	0.54	0.4							
ang	0.32	0.33	0.32	0.42	0.41	0.42	0.4	0.5	0.41	0.41	0.38	0.4						
brad	0.33	0.34	0.33	0.38	0.41	0.41	0.38	0.43	0.39	0.4	0.38	0.37	0.5					
Nfp	0.30	0.31	0.29	0.41	0.37	0.34	0.37	0.45	0.33	0.31	0.35	0.32	0.47	0.42				
∂-op	0.30	0.31	0.31	0.40	0.39	0.41	0.44	0.42	0.38	0.35	0.4	0.38	0.47	0.43	0.46			
som	0.30	0.32	0.34	0.45	0.44	0.47	0.42	0.47	0.43	0.42	0.42	0.38	0.47	0.45	0.47	0.6		
vip	0.36	0.34	0.37	0.47	0.44	0.46	0.45	0.58	0.41	0.49	0.45	0.47	0.59	0.48	0.52	0.48	0.51	

unexpected results.

Chang *et al* (1993) suggest that the 'secretin' subgroup also includes the bombesin and VIP receptors, as they show by peptide sequence analysis. However, in this analysis no such grouping was obvious, even at TM regions.

The actual sequence alignments for each group as determined in Table 2.46 are shown in Appendix II. If these figures are studied it can be seen how the consensus sequence varies from group to group in each case providing a unique peptide sequence specific for that group. From these sequences PCR primers were designed, with the aim of discovering new receptors which would cluster, by sequence, with that sub-group.

By initially selecting groups with high homology (high pairwise alignment scores) and choosing regions in the consensus which are highly conserved and unique to that sub-group, primers can be designed which are selective for that group and with the introduction of limited degeneracy also capable of amplifying related receptors with similar sequence conservation.

Table 2.41 Receptors grouped by homology at TM 1

i	ii	iii	iv
parathyroid hormone	neurokinin 1	neurotensin	vasoactive intestinal peptide
secretin	neurokinin 2	endothelin B	δ opioid
calcitonin	neurokinin 3	cholecystokinin	somatostatin
	bombesin	neuropeptide Y	thyrotropin releasing hormone
		N-formyl peptide	
		angiotensin 2	
		bradykinin 3	

Table 2.42 Receptors grouped by homology at TM 2

i	ii	iii	iv	v
parathyroid hormone	neurokinin 1	neurotensin	endothelin B	neuropeptide Y
secretin	neurokinin 2	thyrotropin releasing hormone	cholecystokinin	N-formyl peptide
calcitonin	neurokinin 3		bombesin	angiotensin 2
				bradykinin 3
				vasoactive intestinal peptide
				δ opioid
				somatostatin

Table 2.43 Receptors grouped by homology at TM 3

i	ii	iii	iv	v
parathyroid hormone	neurokinin 1	neurotensin	bombesin	N-formyl peptide
secretin	neurokinin 2		endothelin B	angiotensin 2
calcitonin	neurokinin 3		cholecystokinin	bradykinin 3
	thyrotropin releasing hormone		neuropeptide Y	vasoactive intestinal peptide
				δ opioid
				somatostatin

Table 2.44 Receptors grouped by homology at TM6

i	ii	iii	iv	v
parathyroid hormone	neurokinin 1	bombesin	N-formyl peptide	vasoactive intestinal peptide
secretin	neurokinin 2	endothelin B	angiotensin 2	somatostatin
calcitonin	neurokinin 3	cholecystokinin	bradykinin 3	
	thyrotropin releasing hormone	neuropeptide Y		
	neurotensin			
	δ opioid			

Table 2.45 Receptors grouped by homology at TM7

i	ii	iii	iv	v	vi
secretin	neurokinin 1	neurotensin	bombesin	angiotensin 2	δ opioid
calcitonin	neurokinin 2	neuropeptide Y	endothelin B	bradykinin 3	somatostatin
parathyroid hormone	neurokinin 3	thyrotropin releasing hormone	cholecystokinin	N-formyl peptide	vasoactive intestinal peptide

Table 2.46 Receptors grouped by overall homology

i	ii	iii	iv	v	vi
secretin	neurokinin 1	neurotensin	bombesin	angiotensin 2	δ opioid
calcitonin	neurokinin 2	neuropeptide Y	cholecystokinin	bradykinin 3	somatostatin
parathyroid hormone	neurokinin 3	thyrotropin releasing hormone	endothelin	N-formyl peptide	vasoactive intestinal peptide

2.5 Polymerase Chain Reaction

Tables 2.51-2.54 show the sequences of the primers designed as a result of the sequence analysis work, and Tables 2.55 & 2.56 the thermal cycling conditions that they were used at. Figure 2.50 & 2.51 (p36a-d) show the primer alignments.

These particular receptor groups were chosen as they were the most distinctive sets from the entire sequence comparison, always clustering together, and this close sequence homology allowed the design of degenerate primers with minimal complexity, so that they retained some specificity.

Table 2.51 Mouse neurokinin-1 receptor control primers

primer name	5' primer sequence 3'	length bp	TM	T_m	sense
MNK1-3	tatcgc**ggtacc**TGCAAGTTTCACAATTCTTCCC	35	3	66°C	sense
MNK1-7	tatcgc**ggtacc**CCACATGCTGGCCAGGTAGACCT	35	7	74°C	anti

Table 2.52 Tachykinin group degenerate primers, based on the alignment of sequences in Table 2.46, group ii

primer name	5' primer sequence 3'	length bp	TM	degeneracy	T_m
TACH-3	tatcgc**ggtacc**GC**Y** **S**TS TTCG**Y** C**W** **S** **Y**ATITACTC	35	3	$2^7 = 128$	64-70°C
TACH-7	tatcgc**ggtacc**CCAIA**W**I**S** **K**IGCCA**R** **R**AT**S** ACCT	35	7	$2^6 = 64$	58-62°C

Table 2.53 Rat parathyroid hormone receptor control primers

primer name	5' primer sequence 3'	length bp	TM	T_m	sense
PATH-3	tatcgc**ggtacc**AACTACTACTGGATTCTGGTGGAG	36	3	70°C	sense
PATH-7	tatcgc**ggtacc**GCCTGCACCTCACCATTGCAGAA	36	7	72°C	anti

Figure 2.50 Alignment of mouse tachykinin receptors showing the sites at which the primers were designed.

```
     1                                                            50
nk1  ~~~~~~~~~~ ~~~TTGCTGC CTTGCCCGCC AAAATGGATA ACGTCCTTCC
nk2  AGACCCTGTG TTCCAGGCCC AGGATCAGCC ATGGGGGCCC ACGCCAGCGT
nk3  ATCACTGAGT GGCTTGCGCT CCAGGCTGGC AACTTCTCCT CCGCGCTGGG

                                                            100
nk1  TGTGGACTCT GATCTCTTCC CCAACACCTC CACCAACACT TCTGAGTCTA
nk2  TACCGACACC AACATCTTGT CTGGCCTTGA GAGTAACGCA ACAGGCGTTA
nk3  CTTGCCAGTG ACATCCCAGG CACCTTCCCA AGTCCGGGAC AACCTGACGA

                                                            150
nk1  ACCAGTTTGT GCAACCTACC TGGCAAATTG TCCTTTGGGC AGCCGCCTAT
nk2  CAGCCTTCTC TATGCCTGGC TGGCAGCTGG CGCTATGGGC CACAGCCTAC
nk3  ACCAGTTCGT GCAGCCCTCC TGGCGAATCG CGCTCTGGTC GCTGGCCTAT

                                                            200
nk1  ACGGTCATCG TGGTGACTTC CGTGGTGGGC AACGTAGTGG TGATATGGAT
nk2  CTGGCCCTGG TGCTGGTGGC TGTAACAGGC AACGCCACAG TCATCTGGAT
nk3  GGCTTAGTGG TGGCGGTGGC AGTCTTCGGA AACCTCATCG TTATTTGGAT
                                TM1
                                                            250
nk1  CATTTTGGCC CACAAGAGAA TGAGGACAGT GACCAATTAT TTCCTGGTGA
nk2  CATTCTGGCC CATGAGAGAA TGCGCACGGT CACCAACTAT TTCATCATCA
nk3  CATCTTGGCC CACAAGCGCA TGAGGACCGT CACCAACTAT TTCCTGGTAA

                                                            300
nk1  ACCTGGCCTT CGCTGAGGCC TGCATGGCTG CATTCAATAC AGTGGTGAAC
nk2  ACCTGGCCTT GGCAGACCTC TGCATGGCGG CCTTCAATGC CACCTTCAAC
nk3  ACCTGGCTTT CTCCGACGCC TCCGTGGCTG CCTTCAACAC CTTGGTCAAT
                                TM2
                                                            350
nk1  TTCACCTACG CAGTCCACAA CGTGTGGTAC TACGGCCTCT TTTACTGCAA
nk2  TTCATCTATG CCAGTCACAA CATCTGGTAC TTCGGCAGCA CCTTCTGCTA
nk3  TTCATCTATG GTGTTCACAG CGAGTGGTAC TTTGGCGCCA ACTACTGCCG

          mnk1-3                                            400
nk1  GTTTCACAAC TTCTTCCCCA TTGCTGCTCT CTTCGCCAGT ATCTACTCCA
nk2  CTTCCAGAAC CTCTTTCCTG TCACAGCCAT GTTCGTCAGC ATCTACTCCA
nk3  CTTCCAGAAC TTCTTTCCCA TCACAGCGGT GTTTGCCAGC ATCTACTCTA
                                TM3          tach-3
                                                            450
nk1  TGACAGCTGT GGCCTTTGAC AGATACATGG CCATCATCCA CCCTCTTCAG
nk2  TGACCGCCAT CGCCGCTGAC AGGTACATGG CCATTGTCCA CCCTTTCCAG
nk3  TGACAGCCAT TGCAGTGGAC AGGTATATGG CCATTATCGA TCCTTTGAAA

                                                            500
nk1  CCCCGGCTGT CGGCCACTGC TACCAAAGTG GTCATCTTTG TCATCTGGGT
nk2  CCACGGCTCT CCGCCCCCAG CACCAAGGCG GTTATTGCTG TCATCTGGCT
nk3  CCCAGACTAT CTGCCACAGC CACTAAGATT GTCATCGGAA GTATTTGGAT

                                                            550
nk1  CCTGGCTCTC CTGCTGGCCT TTCCACAGGG CTACTACTCC ACCACAGAGA
nk2  GGTAGCCCTG GCTCTCGCCT CCCCACAATG TTTCTACTCC ACCATCACTG
nk3  TTTGGCATTT CTACTTGCCT TCCCTCAATG TCTTTATTCC AAAATAAAAG
                                TM4
```

```
                                                                    600
nk1  CCATGCCCAG CAGAGTAGTG TGCATGATAG AGTGGCCAGA ACATCCCAAC
nk2  TGGACCAGGG GGCCACCAAG TGTGTGGTGG CCTGGCCCAA TGACAACGGA
nk3  TCATGCCAGG CCGTACCCTT TGCTATGTGC AGTGGCCAGA AGGTCCCAAG

                                                                    650
nk1  AGGACTTACG AGAAAGCGTA CCACATCTGT GTGACTGTGC TGATCTACTT
nk2  GGCAAGATGC TCCTACTGTA TCATCTGGTG GTGTTTGTCC TCATCTACTT
nk3  CAACATTTC. .....ACGTA CCACATCATT GTTATCATCC TGGTGTACTG

                                                                    700
nk1  CCTGCCTCTG CTGGTGATTG GCTATGCATA CACTGTGGTA GGGATTACAC
nk2  CCTGCCTCTA GTGGTGATGT TTGCAGCTTA CAGTGTCATT GGCCTCACAC
nk3  TTTCCCATTG CTCATCATGG GTGTCACCTA CACCATCGTT GGAATTACTC
                                  TM5
                                                                    750
nk1  TGTGGGCCAG TGAGATCCCC GGTGAC...T CCTCTGACCG TTACCATGAG
nk2  TGTGGAAACG CGCCGTACCC AGACACCAGG CTCATGGAGC TAACCTGCGC
nk3  TCTGGGGAGG AGAGATCCTA GGAGAC...A CCTGTGACAA GTACCATGAG

                                                                    800
nk1  CAAGTCTCTG CCAAGCGCAA GGTGGTCAAA ATGATGATCG TGGTTGTGTG
nk2  CATCTACAGG CCAAGAAGAA GTTTGTGAAG GCCATGGTAC TGGTGGTGGT
nk3  CAGCTTAAGG CTAAACGAAA GGTTGTAAAA ATGATGATTA TTGTGGTGGT

                                                                    850
nk1  TACCTTCGCC ATCTGCTGGC TGCCCTTCCA CATCTTCTTC CTCCTGCCCT
nk2  GACATTTGCC ATCTGCTGGC TGCCCTACCA CCTCTACTTC ATCCTGGGGA
nk3  GACATTTGCC ATCTGCTGGC TACCCTACCA TGTGTATTTC ATTCTCACTG
                                  TM6
                                                                    900
nk1  ACATCAACCC AGATCTCTAC CTTAAGAAGT TCATCCAGCA GGTCTACCTG
nk2  CCTTCCAAGA GGACATCTAC TACCGCAAGT TTATCCAGCA GGTCTACCTG
nk3  CGATCTACCA ACAGTTAAAC AGGTGGAAAT ACATCCAGCA GGTCTACCTG
                                                        tach-7
     mnk1-7                                                         950
nk1  GCCAGCATGT GGCTGGCCAT GAGTTCTACC ATGTACAACC CCATCATCTA
nk2  GCACTCTTCT GGCTGGCCAT GAGTTCCACC ATGTACAACC CCATCATTTA
nk3  GCTAGCTTCT GGCTGGCCAT GAGCTCAACC ATGTACAACC CCATCATCTA
                                  TM7
                                                                   1000
nk1  CTGCTGCCTC AATGACAGGT TCCGTCTGGG CTTCAAGCAT GCCTTTCGCT
nk2  TTGCTGCCTT AACCACAGGT TTCGCTCTGG ATTCCGGCTT GCTTTCCGGT
nk3  CTGCTGTTTG AACAAAAGAT TTCGTGCAGG CTTCAAGAGA GCATTTCGCT

                                                                   1050
nk1  GCTGCCCTTT CATCAGTGCT GGTGATTATG AGGGGCTGGA AATGAAATCC
nk2  GCTGCCCCTG GGGGACACCA ACCGAGGAAG ACAGGCTGGA GCTGACCCAC
nk3  GGTGTCCTTT CATCCAAGTC TCCAGCTACG ATGAGCTGGA GCTCAAGACC

                                                                   1100
nk1  ACCCGATACC TCCAGACCCA GAGCAGCGTG TACAAGGTCA GCCGCCTGGA
nk2  ACTCCGTCCA TCTCCAGGAG AGTCAACCGG TGTCACACCA AGGAGACTTT
nk3  ACCAGGTTTC ATCCCACACG GCAGCCA~~~ ~~~~~~~~~~ ~~~~~~~~~~

                                                                   1150
nk1  GACCACCATC TCCACTGTGG TGGGAGCCCA TGAAGATGAG CCAGAGGAAG
nk2  GTTCATGACA GGGGATATGA CCCACTCTGA GGCTACCAAT GGGCAGGTTG
nk3  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
```

TM domains are labelled and indicated by a single underline.
Primers are labelled and indicated by a double underline.

Figure 2.5 05 Rat parathyroid hormone receptor sequence showing the points at which the primers align.

```
         ---------- ---------- --ATGGGGGC CGCCCGGATC GCACCCAGCC
    29   TGGCGCTCCT ACTCTGCTGC CCAGTGCTCA GCTCCGCATA TGCGCTGGTG
    79   GATGCGGACG ATGTCTTTAC CAAAGAGGAA CAGATTTTCC TGCTGCACCG
   129   TGCCCAGGCG CAATGTGACA AGCTGCTCAA GGAAGTTCTG CACACAGCAG
   179   CCAACATAAT GGAGTCAGAC AAGGGCTGGA CACCAGCATC TACGTCAGGG
   229   AAGCCCAGGA AAGAGAAGGC ATCGGGAAAG TTCTACCCTG AGTCTAAAGA
   279   GAACAAGGAC GTGCCCACCG GCAGCAGGCG CAGAGGGCGT CCCTGTCTGC
   329   CCGAGTGGGA CAACATCGTT TGCTGGCCAT TAGGGGCACC AGGTGAAGTG
   379   GTGGCAGTAC CTTGTCCCGA TTACATTTAT GACTTCAATC ACAAAGGCCA
   429   TGCCTACAGA CGCTGTGACC GCAATGGCAG CTGGGAGGTG GTTCCAGGGC
   479   ACAACCGGAC GTGGGCCAAC TACAGCGAGT GCCTCAAGTT CATGACCAAT
   529   GAGACGCGGG AACGGGAGGT ATTTGACCGC CTAGGCATGA TCTACACCGT
                                                        TM1
   579   GGGATACTCC ATGTCTCTCG CCTCCCTCAC GGTGGCTGTG CTCATCCTGG
   629   CCTATTTTAG GCGGCTGCAC TGCACGCGCA ACTACATCCA CATGCACATG
                                                        TM2
   679   TTCCTGTCGT TTATGCTGCG CGCCGCGAGC ATCTTCGTGA AGGACGCTGT
   729   GCTCTACTCT GGCTTCACGC TGGATGAGGC CGAGCGCCTC ACAGAGGAAG
   779   AGTTGCACAT CATCGCGCAG GTGCCACCTC CGCCGGCCGC TGCCGCCGTA
   829   GGCTACGCTG GCTGCCGCGT GGCGGTGACC TTCTTCCTCT ACTTCCTGGC
   879   TACCAACTAC TACTGGATTC TGGTGGAGGG GCTGTACTTG CACAGCCTCA
                          path-3                    TM3
   929   TCTTCATGGC CTTTTTCTCA GAGAAGAAGT ACCTGTGGGG CTTCACCATC
   979   TTTGGCTGGG GTCTACCGGC TGTCTTCGTG GCTGTGTGGG TCGGTGTCAG
                                                        TM4
  1029   AGCAACCTTG GCCAACACTG GGTGCTGGGA TCTGAGCTCC GGGCACAAGA
  1079   AGTGGATCAT CCAGGTGCCC ATCCTGGCAT CTGTTGTGCT CAACTTCATC
                                                        TM5
  1129   CTTTTTATCA ACATCATCCG GGTGCTTGCC ACTAAGCTTC GGGAGACCAA
  1179   TGCGGGCCGG TGTGACACCA GGCAGCAGTA CCGGAAGCTG CTCAGGTCCA
  1229   CGTTGGTGCT CGTGCCGCTC TTTGGTGTCC ACTACACCGT CTTCATGGCC
                                                        TM6
  1279   TTGCCGTACA CCGAGGTCTC AGGGACATTG TGGCAGATCC AGATGCATTA
  1329   TGAGATGCTC TTCAACTCCT TCCAGGGATT TTTTGTTGCC ATCATATACT
                                                        TM7
```

1379 GTTTCTGCAA TGGTGAGGTG CAGGCAGAGA TTAGGAAGTC ATGGAGCCGC

path-7

1429 TGGACACTGG CGTTGGACTT CAAGCGCAAA GCACGAAGTG GGAGTAGCAG

1479 CTACAGCTAT GGCCCAATGG TGTCTCACAC GAGTGTGACC AATGTGGGCC

1529 CCCGTGCAGG ACTCAGCCTC CCCCTCAGCC CCCGCCTGCC TCCTGCCACT

1579 ACCAATGGCC ACTCCCAGCT GCCTGGCCAT GCCAAGCCAG GGGCTCCAGC

1629 CACTGAGACT GAAACCCTAC CAGTCACTAT GGCGGTTCCC AAGGACGATG

1679 GATTCCTTAA CGGCTCCTGC TCAGGCCTGG ATGAGGAGGC CTCCGGGTCT

1729 GCGCGGCCGC CTCCATTGTT GCAGGAAGAA TGGGAAACAG TCATG-----

Tm domains are labelled and indicated by a single underline
Primers are labelled and souble underlined

Table 2.54 Secretin group degenerate primers, based on the alignment of sequences in Table 2.46, group i

primer name	5' primer sequence 3'	length bp	TM	degeneracy	T_m
SEC-3	tatcgc**ggtacc**AACTA**Y**K**I**M**T**GGM**T**I**C**T**S**K**K**K**G	34	3	$2^8 = 256$	56-66°C
SEC-7	tatcgc**ggtacc**ITGS ACCTCI**Y Y R**TTI**M R**GAA	33	7	$2^6 = 64$	58-62°C

Key: TM -transmembrane domain
T_m -melting temperature
degeneracy -number of different unique primer sequences present
lower case -non-homologous sequence, bold type indicates *KpnI* site
uppercase -homologous sequence, bold type indicates degeneracy

Codes for nucleotide sequence degeneracy:-
M = A or C R = A or G W =A or T S = G or C K = G or T
Y = C or T D = A, G or T H = A, C or T N = A, C, G or T

Tables 2.55-2.56 Thermal cycling conditions

2.55 Amplification using mouse NK-1 receptor control primers

temperature	94°C	94°C	60°C	72°C	4°C
time (minutes)	1	1	2	3	storage
		30 cycles			

2.56 Amplification using degenerate tachykinin receptor primers

temperature	94°C	94°C	55°C	72°C	4°C
time (minutes)	1	1	2	3	storage
		30 cycles			

For the parathyroid hormone receptor (PHR) control primers and degenerate secretin group primers, various annealing temperatures ranging from 50-65°C were tried but they failed to amplify the correct product. In the case of the degenerate primers no products were obtained. Therefore the work was concentrated on the NK-1 receptor primers and the degenerate tachykinin receptor primers.

The control primers were non-degenerate primer pairs of approximately equivalent size and position as the degenerate pairs, but designed towards one member of a group in each case (ie the NK-1 and PH receptors). It was hoped that the use of these primers would allow reaction conditions to be optimised before introducing the added complication of degeneracy.

2.6 PCR Results

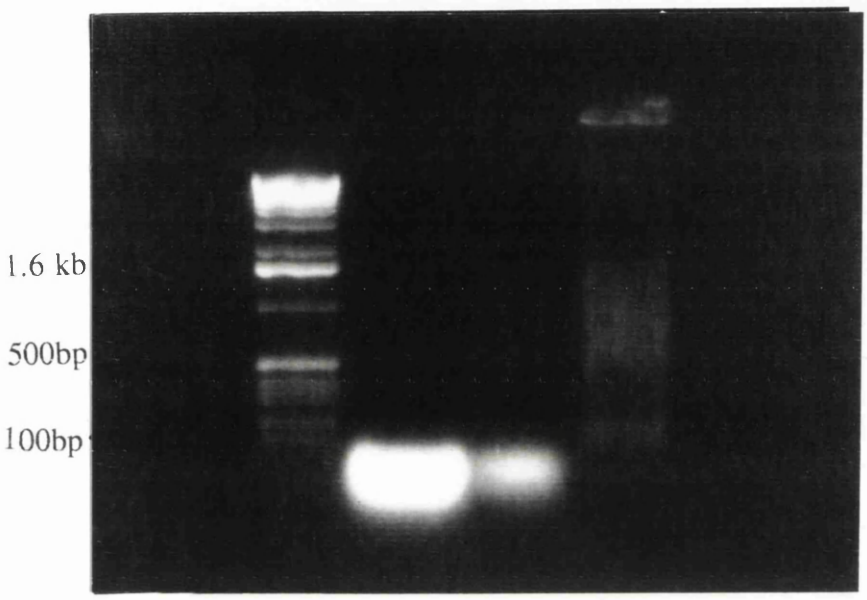
PCR reactions were carried out as in the 'Methods' Chapter 8, section 8.6, p96. Figure 2.61 shows the result of an initial optimisation experiment. No amplification occurred as the annealing temperature was too high and also the concentrations of primer used were 10 fold too high. Reaction conditions were altered accordingly. The Figure also shows 10ng of striatal template cDNA which appears to span a good size range and not be degraded.

In Figure 2.62 (p38b) the template was tested using an established, successful primer pair. Primers for the α-subunit of the $GABA_A$ receptor gene (designed by Dr D Livingstone) were used, at the conditions in Table 2.56 (p37), as a positive control amplifying a 480bp product.

Figure 2.63 (p38c) displays the result of successful amplification with the NK-1 receptor control primers, at the thermal cycling conditions indicated. The product was of the correct size (≈563bp) and cloning and sequencing confirmed its identity. The use of the $GABA_A$ primers here shows the difference between the striatal and cortex template, with the striatal template giving a much stronger band, reflecting the relative mRNA abundance.

Once reaction conditions had been established using the control primers the degenerate primers were used with the same conditions but at successively lower temperatures. At 55°C a small amount of product was visible of about 550bp, a

Figure 2.61 Optimising reaction conditions: Assessing primer concentration and confirmation of cDNA product.



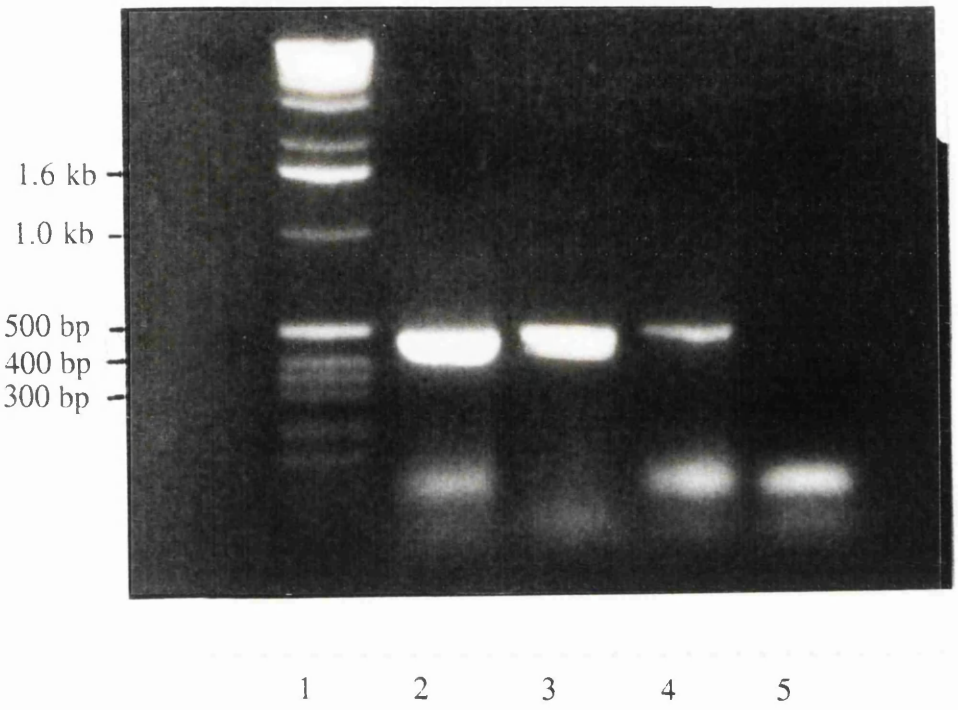
Key to lanes
1 250ng 1kb marker (Gibco BRL)
2 neurokinin-1 primers at 2μM on mouse striatal cDNA
3 neurokinin-1 primers at 1μM on mouse cortex cDNA
4 2μl striatal cDNA (10 ng)

Products were electrophoresed on a 1% agarose gel at 50V for 1 hour

PCR Programme

94°C - 1 min
66°C - 2 min x 30 cycles
72°C - 3 min

Figure 2.62 Confirmation of template quality and reaction conditions: Specific amplification of $GABA_A$ α-subunit fragment by $GABA_{A\alpha}$ specific primers

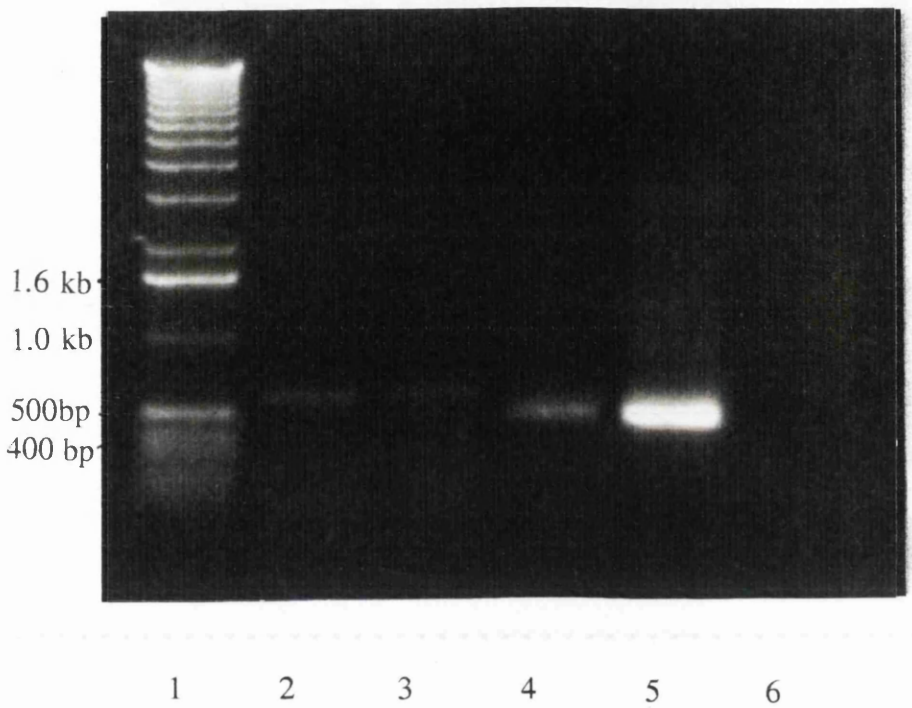


Key to lanes
1 250ng 1kb marker (Gibco BRL)
2 $GABA_{A\alpha}$ primers on mouse striatal cDNA
3 $GABA_{A\alpha}$ primers on mouse striatal cDNA
4 $GABA_{A\alpha}$ primers on mouse cortex cDNA
5 $GABA_{A\alpha}$ primers with no template (negative control)

Products were electrophoresed on a 1% agarose gel at 50V for 1 hour

PCR Programme
95°C - 4 min
⇓
95°C - 1 min
55°C - 2 min x 30 cycles
72°C - 2 min
⇓
4°C soak

Figure 2.63 Positive PCR controls at optimised conditions: products produced by primers designed towards the mouse neurokinin-1 receptor and the α-subunit of the $GABA_A$ receptor gene.



Key to lanes

1	250ng 1kb marker (Gibco BRL)
2	neurokinin-1 primers on mouse striatal cDNA
3	neurokinin-1 primers on mouse cortex cDNA
4	$GABA_{A\alpha}$ primers on mouse striatal cDNA
5	$GABA_{A\alpha}$ primers on $GABA_{A\alpha}$ clone DNA
6	$GABA_{A\alpha}$ primers on mouse cortex cDNA

Products were electrophoresed on a 1% agarose gel at 50 volts for 1 hour

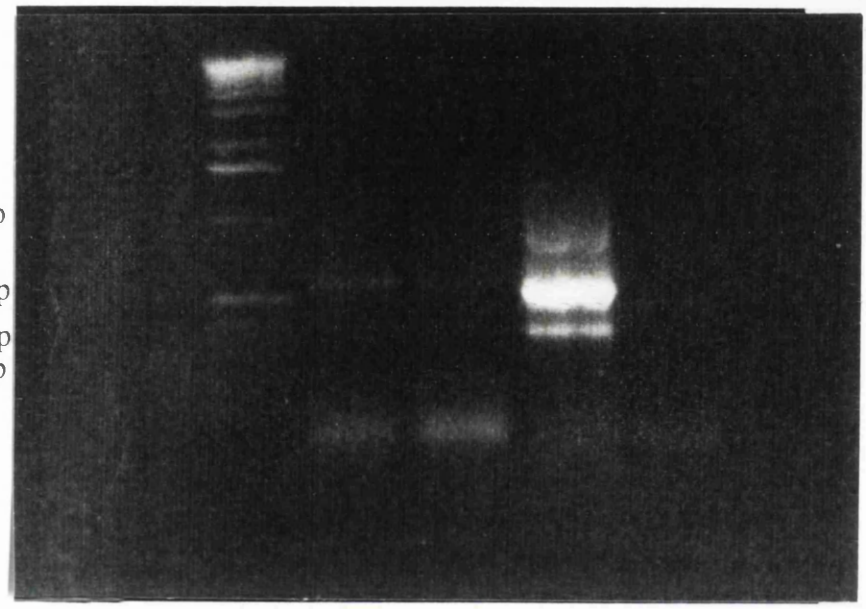
PCR Programme

94°C - 1 min
60°C - 2 min x 30 cycles
72°C - 3 min

Expected product sizes

neurokinin-1 fragment	563 bp
$GABA_A$ fragment	480 bp

Figure 2.64 Products of PCR using the neurokinin-1 control and degenerae tachykinin primer pairs with striatal cDNA template.



Key to lanes

1	250ng 1kb marker (Gibco BRL)
2	neurokinin-1 primers on mouse striatal cDNA
3	tachykinin primers on mouse striatal cDNA
4	tachykinin primers on mouse striatal cDNA (second round PCR products)
5	tachykinin primers with no template (negative control)

PCR Programme

94°C - 1 min
⇓
94°C - 1 min
55°C - 2 min x 30 cycles
72°C - 2 min
⇓
4°C soak

second round at the same conditions using 1µl of the first reaction produced a stronger band (Figure 2.64, p38d). The reaction produced three major products of approximately 800, 550 and 350bp which were individually cloned and sequenced (Chapter 8). Very few clones were obtained for the larger and smaller bands, and sequencing did not produce anything related to the tachykinin receptors. The larger product matched the database sequence for the Prp gene from the Golden Syrian hamster (GenEMBL, accession no. J02686) whilst the sequence from smaller product matched that from the mouse PAP homologous protein gene (GenEMBL, accession no. D13509), (Figures 2.66 & 2.67, p41a). These non-specific products probably arose due to the degenerate nature of the primers allowing these genes to be amplified.

The product at ~550bp proved to be the NK-1 receptor (Figure 2.68, p41b) and no new receptors were isolated.

2.65 Lack of novel products using tachykinin degenerate primers

The PCR reactions were repeated many times at lower annealing temperatures however no new products were obtained. These preliminary experiments suggest that either there are no close relatives of the tachykinin receptors or that the primers used could not detect them. Nevertheless, the general technology was shown to work well with the technique amplifying original members of the group.

SECTION A

CHAPTER 3

DISCUSSION

3.0 Discussion

Sequence analysis

From the Tables 2.41-2.45 (p34 -35) showing grouping of neuropeptide receptors according to peptide sequence, it can be seen that the homology at transmembrane domain seven (TM7) (Table 2.45, p35) best represents the overall homology between these peptide receptors (Table 2.46, p35), but that the other transmembrane regions give different results.

It appears that further towards the 3' end of the sequence there are more group distinguishing motifs. At TM1 the sequences divide into only 4 main groups, at TM2, TM3 & TM5 they can be distinguished into 5 groups and TM7 sequences split the receptors into 6 groups. Six groups can also be defined from the analysis of the whole sequences. Also the group allocation varies between TM domains, eg for the thyrotropin releasing hormone receptor (TRH). It groups with the somatostatin, VIP and δ-opioid receptors at TM1, with neurotensin at TM2, and the tachykinin receptors at TM3 & 6. however, the overall homology agrees with the grouping at TM7 along with the neurotensin and neuropeptide Y receptors.

This result suggests that analysing the homology at the TM regions alone could give a false impression of the subgroups within the neuropeptide receptor family.

The results here contrast with previous classification; for example Chang *et al* (1993) suggest that the 'secretin' subgroup also includes the bombesin and VIP receptors, as they show by peptide sequence analysis. However, in this analysis no such grouping was obvious, even at TM regions. This could possibly be explained if Chang *et al* used only a few sequences in their comparison. A reasonable amount of homology exists between all GPLRs, no matter how distantly related, and therefore any selection of GPLRs once aligned will have

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some points of homology. However, the study of a larger range of receptor sequences using alignment and statistical analysis of the homology actually indicates a different, and perhaps more accurate conclusion of the true sub-groups.

To allocate groups by sequence homology seems a reasonable system. The groups indicate evolutionary relationships, as those with a higher degree of sequence homology are more likely to have diverged from a more recent common ancestor than those with less homology. Also those groups which have little or no sequence homology, but have structural homology as members of the GPLR superfamily, may have convergently evolved.

This study illustrates a simple an definitive method of receptor subgroup allocation by amino acid sequence homology.

2.7 Polymerase Chain Reaction

The amplification of the control fragments had a limited success. In the case of the neurokinin-1 receptor (NK-1R) the primers were successful. The reaction was able to be optimised and the product cloned and confirmed by sequence analysis. This optimisation was aided by the use of a positive control primer pair towards the $GABA_A$ receptor, previously designed and successfully used by Dr David Livingstone.

Once it had been confirmed that the NK-1 receptor had been amplified, equivalent conditions were used for the degenerate primers for this group of receptors (TACH-3 & TACH-7). Initially this produced a very low level of product which may have been due to the successful primer species being at a lower concentration in the population of primers. However, a second round of PCR using the initial product as template produced three individual products. One appeared to be the same size as the NK-1R product (563bp); the second larger at about 800bp and the

Figure 2.71 *Alignment of large PCR product to Golden Syrian Hamster Prp gene (J02686), result of a 'Fasta' search on GCG. The sequences have 83.4% identity over a 175 bp overlap*

```
              60          70          80          90          100
Seqrta CA--CT-CGC-ACTGTTGG--AAGGCGATCGGTGCGGG-CTCTTCGCGATT--GCAGCTG
       ||  || ||| ||||||||  | ||||||||||||||| ||||||||  |||   ||||||
Hamprp CAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTAGCCAGCTG
      4720       4730       4740       4750       4760       4770

         110         120         130         140         150         160
Seqrta GCGAAA-GGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCA
       |||||| |||||||||||| |||||||||||||||| ||||| ||||||||||||||||
Hamprp GCGAAAGGGGGATGTGCTGTAAGGCGATTAAGTTGGGCAACGCTAGGGTTTTCCCAGTCA
      4780       4790       4800       4810       4820       4830

            170         180         190         200         210         220
Seqrta CGACG-TGTAAAACGACGGCCAGTGAGCTGCGTAATACGACTCACTATAGGGCGAATTGG
       ||||| ||||||||||||||||||
Hamprp CGACGTTGTAAAACGACGGCCAGTG
      4840       4850       4860
```

Figure 2.72 *Alignment of small PCR product to mouse PAP homologous protein gene (D13509), result of a 'Fasta' search on GCG. The sequences have 87.1% identity over a 155 bp overlap*

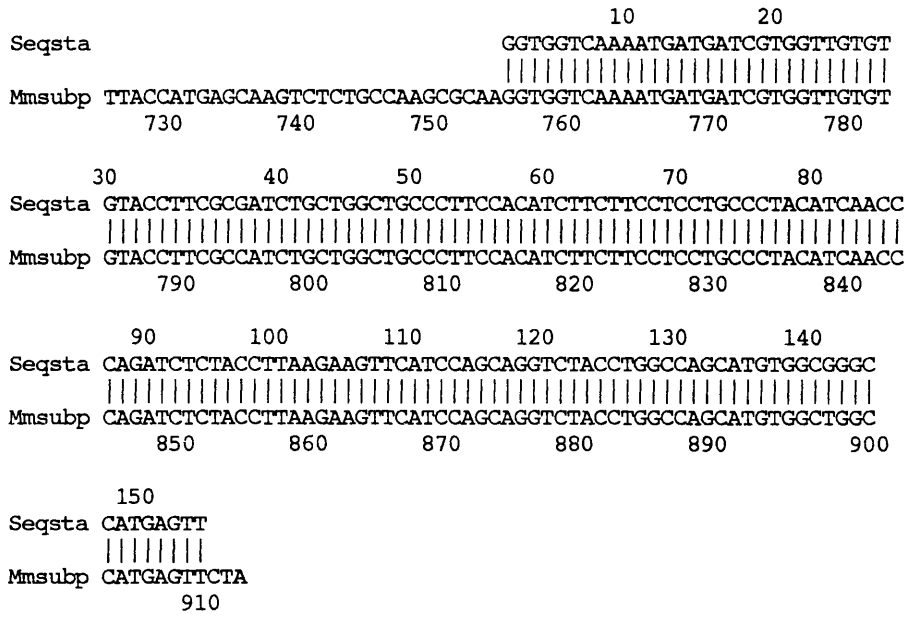
```
              30          40          50          60          70          80
Seqrta AGGCCACAGCTGTCATGGAGTACATGCAGACGAACACGGCGGTAC-CCAATTCGCCCTAT
                                       | | | ||| ||  | ||||||||||||||
Mmpapl CCGGGGGATCCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTCCAATTCGCCCTAT
         570         580         590         600         610         620

               90         100         110         120         130
Seqrta AGTGAGTCGTATTACGCAGCTCACTGGCCGTCGTTTTAC-ACGTCGTGACTGGGAAAACC
       |||||||||||||  ||  ||||||||||||||||||| |||||||||||||||||||||
Mmpapl AGTGAGTCGTATTA--CAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACC
         630         640         650         660         670         680

     140         150         160         170         180          190
Seqrta CTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCTTTCGCCAGCT---GCAATCG
       |||||||||||||||||||||||||  ||||||||||||||||||||   | ||| |
Mmpapl CTGGCGTTACCCAACTTAATCGCCTT---GCACATCCCCTTTCGCCAGCTGGCGTAATAG
           690         700          710         720         730         740

         200         210         220         230         240         250
Seqrta CGAAGAGCCCGCACCGATCGCCTTCCAACAGTGCGAGTGAATGCAATGAATGTAGCGTTA
       |||||
Mmpapl CGAAG
```

Figure 2.68 *Alignment of the 563bp PCR product to NK-1 receptor gene. The sequences have 100% identity over their entire length.*



third smaller at approximately 350bp.

On further analysis the sequences revealed that the 563bp product was indeed NK-1R (Figure 2.73, p41b) but the other two products were not related to tachykinin receptors. The larger product matched the database sequence for the Prp gene from the Golden Syrian hamster (GenEMBL, accession no. J02686) whilst the sequence from smaller product matched that from the mouse PAP homologous protein gene (GenEMBL, accession no. D13509), (Figures 2.71 & 2.72, p41a). These non-specific products probably arose due to the degenerate nature of the primers and also the high number of cycles created by performing a secondary round of PCR (Innis & Gelfand,1990).

As these primers had given these initially promising results, the next step was to alter the conditions so as to increase the probability of amplifying a related receptor. To use slower ramping times between the annealing and extension temperatures and to perform the initial 2-5 cycles at a low nonstringent annealing temperature. These conditions should allow a greater proportion of the population degenerate of primers to anneal, therefore yeilding a greater number of products, which when screened could include a new tachykinin receptor group member (Compton, 1990).

Work on this optimisation was started, however, being an MSc project with a limited time-span it was decided to shift the focus of to an area which would give guaranteed results. The D_2-like dopamine receptors were of considerable interest to my industrial sponsors and therefore it was decided to concentrate on this group by cloning and further characterising these receptors.

At the time that this research began this was a rapidly expanding area with evidence for many GPLR receptors as yet uncloned. Although research has advanced greatly since this time, the work contained in this project is still of interest and use in currect research. The method of sub-grouping receptors by sequence and subsequently using the consensus produced for degenerate primer design has great potential in the discovery of new GPLRs.

SECTION B

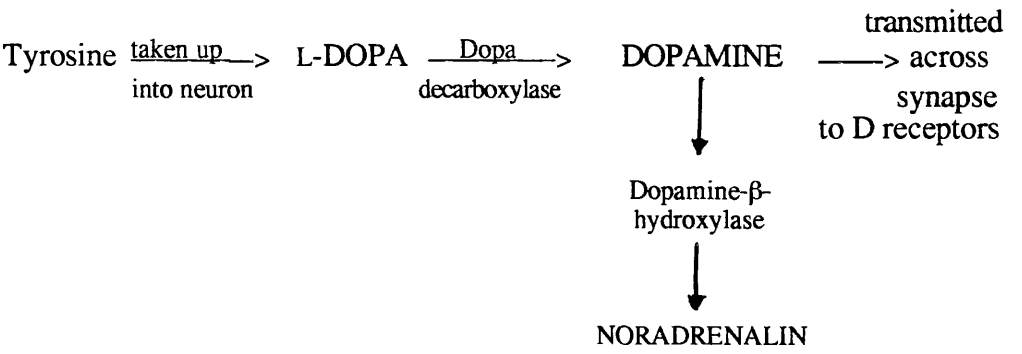
CHAPTER 4

INTRODUCTION

Investigation of Dopamine D_{2S} Receptors

The cationic amine dopamine is an intermediate in the biosynthetic pathway of the neurotransmitters adrenaline and noradrenaline, it is also an important neurotransmitter itself although it is much less widely distributed in brain tissue. Dopamine neurons lack the enzyme dopamine β-hydroxylase and therefore dopamine is the final product of the pathway.

Figure 4.01 Synthesis of Dopamine



In the central nervous system (CNS) dopamine is involved in the control of motor, cognitive, affective and neuroendocrine processes and in the peripheral nervous system dopamine regulates hormone synthesis and secretion, vascular tone and renal function.

Dopaminergic systems are of particular interest because of their role in the aetiology and management of various disorders such as the motor disorders, Parkinson's disease and Huntington's disease. Both involve nigrostriatal degeneration and an alteration in dopamine neurotransmission, decreased in Parkinsonism leading to rigidity, tremor and hypokinesia and in Huntington's an increase causing severe involuntary movements.

In the mesolimbic system which is involved with cognitive and emotional responses, dopamine also acts as a neurotransmitter and it is believed that it may be involved with the etiology of schizophrenia although there has been no evidence of altered transmission.

Most dopamine in the brain is located in the nigrostriatal pathway and the axons extending into the striatum, it is also found to varying extents in the nucleus accumbens, hypthalamus, pituitary gland, olfactory bulb and retina. In the periphery it is present in some sympathetic neurons, mainly in the kidney.

4.1 Dopamine Receptors

Initial research concluded that the effects of dopamine could be accounted for by the existence of two receptor subtypes which were named the D_1 and D_2 dopamine receptors (Kebabian & Calne, 1979). Recent developments however have demonstrated that the family is larger, with six distinct dopamine receptor subtypes isolated at present and indications of further, as yet unidentified members.

4.11 D_2-like Receptors

The rat D_2 receptor was the first member cloned (Bunzow *et al,* 1988) and was confirmed as possessing the functional properties originally predicted by Sasaki & Sato (1987) of coupling inhibitorally to adenylate cyclase. In addition the high affinity for benzamides and low affinity for benzazepines discovered by Stoof & Kebabian (1984) was shown to conform (Albert *et al,* 1990).

The D_2 dopamine receptor is typical of the classic GPLR structure however it has been found to exist in two forms derived from the same gene by alternative splicing (Dal Toso *et al,* 1989). The long form (D_{2L}) contains an additional 29 amino acids in the third cytoplasmic loop and has slight pharmacological

differences from the short form (D_{2S}). The major difference so far reported is that D_{2S} inhibits adenylyl cyclase to a greater extent (Dal Toso *et al,* 1989). The long form predominates in rodents and the short in humans.

Additional 'D_2-like' receptors were subsequently cloned, D_3 (Sokoloff *et al,* 1990; Giros *et al,* 1990) and D_4 (Van Tol *et al,* 1991). Both bind the benzamide and butyrophenone ligands as D_2 does but also possess distinguishing pharmacological characteristics. Structurally they are also similar, having a classic GPLR arrangement, with short C terminal and long I3 loops, characteristic of GPLRs which are coupled to G_i leading to inhibition of adenylyl cyclase and Ca^{2+} channels and activation of K^+ channels.

Subsequently, a second spliced isoform of the D_3 receptor has been isolated and also many variants of the human D_4 receptor. The D_4 receptor varies at the third cytoplasmic loop region and was the first catecholamine receptor to be discovered with polymorphic variation involving the repeat of a 48 base pair sequence from two to seven times ($D_{4.2}$, $D_{4.3}$ etc.), in human populations. Each has a different pharmacological profile for spiperone and clozapine binding (Van Tol *et al,* 1992). The fact that this gene shows polymorphism has implications for neuropsychiatric disease (such as schizophrenia) as other occurances of repeated sequences have been implicated in disease (eg fragile X syndrome and myotonic dystrophy).

4.12 D_1-Like Receptors

Using strategies based on the nucleotide sequence of the cloned D_2 receptor several groups discovered other slightly less homologous dopamine receptors. The first of these was named the D_1 receptor as it conformed well to the expected second messenger coupling and pharmacological properties of the classical D_1 described by Kebabian & Calne 1979. Structurally this receptor also had the GPLR topology, with 40 - 45% homology at the residue level to D_2, it also had

homology to the β-adrenergic and 5HT receptors. (Dearry *et al,* 1990; Monsma *et al,* 1990; Sunhara *et al,* 1990; Zhou *et al,* 1990). The coupling of this receptor to the adenylyl cyclase system was found to be stimulatory via coupling to the Gs protein.

Subsequently other D_1-like dopamine receptors were cloned and characterised from rat and human (Sunhara *et al,* 1991; Tiberi *et al,* 1991). They shared a high TM residue identity of ~80% with the D_1 receptor, the same coupling to Gs and also the absence of introns within their coding regions, as with the D_1 receptor gene. They have patterns of mRNA in the same regions as D_1 but at a 10x lower level of expression but also a high level of expression in the hippocampus, anterior pretectal nuclei and mammalian nuclei (Tiberi *et al,* 1991) unlike D_1.

Problems with inconsistent nomenclature have lead these D_1-like receptors to be known as D_5 or less confusingly D_{1B} with D_1 being renamed D_{1A} (Tiberi *et al,* 1991).

4.2 Importance Of Dopamine Receptor Research

The discovery of new dopamine receptors has been due to the use of molecular biology making it possible to define distinct receptor subtypes previously indistinguishable by biochemical and pharmacological properties. Difficulties such as large variances in the expression of different subtypes and individual expression patterns made further biochemical discovery difficult. However, pharmacology has been vital in providing the initial essential evidence for the existence of such receptors and points to evidence for additional dopamine receptor subtypes.

4.3 Dopamine Receptor Pharmacology

Assessing the binding affinities of certain compounds for receptors is usually performed using radioligands in saturation and competition assays, this is fine for antagonists which block the action of the receptor, however agonists (which stimulate action) are more complex to assess. In tissue, the receptor can be in either of two states, high or low affinity for the agonist. Low affinity receptors are not linked to a G protein, whereas high affinity are which is though to give a conformational change making the receptor more receptive to agonist binding. This situation must be mimicked in vitro to give a true measurement of agonist binding and this is done by the addition of GTP and Mg^{2+} ions, which causes the receptors to dissociate from linked G proteins leaving all in a standard low affinity state.

4.31 D_1-like Receptor Pharmacology

As mentioned previously the D_1 and D_5 receptors share high amino acid homology in the TM domains and as these regions are thought to be involved in ligand binding it therefore follows that these receptors have a very similar ligand binding profile. They both have a high affinity for benzazepines (eg SKF 38393, a partial agonist and SCH 23390 an antagonist) and low affinity for butyrophenones (eg spiperone and halperidol). However there are presently only two notable binding differences, dopamine has a tenfold greater affinity for D_5 (Tiberi *et al,* 1991; Sunahara *et al,* 1991) and the antagonists (+) butaclamol and SKF83566 have greater affinity for D_1 (Gingrich & Caron, 1993).

4.32 D_2-Like Receptor Pharmacology

Of the three full length receptors, D_4 has the most distinctive pharmacological profile, as would be expected as it also has the most differences in sequence at the TM regions.

A general shared characteristic, in contrast to the D_1-like receptors, is that they each have a high affinity for butyrophenones and a low affinity for some

benzazepines (eg SKF 38393. However, D_2 and D_3 have relatively high affinity for raclopride (another benzazepine) whereas D_4 has a much lower affinity. An antagonist which appears to have selectively higher affinity for D_4 is clozapine. There is much debate about whether this is true, but it remains the only selective ligand for separating the D_2-like group members (Van Tol *et al*, 1991). This information is summarised in Table 4.321.

Table 4.321 D_2-Like Selective Ligands

Receptor ⇒ Selective Ligands	D_{2L}	D_3	D_4
Agonists	N-0437 bromocriptine	7-OH-DPAT* quinperole quinerolane	none
Antagonists	(+) butaclamol YM091512 domperidone haloperidol remoxipride	all D2 ligands but with lower affinity eg remoxipride binds with ~100x less affinity	clozapine (binds with a 10x higher affinity than for D2/3)
Radiolabels	[^{3}H]-spiperone (also binds 5HT) [^{3}H]-YM091512 [^{125}I]-rodosulpiride	[^{3}H]-YM091512 [^{3}H]-7-OH-DPAT	[^{3}H]-YM091512 [^{3}H]-clozapine

* 7-hydroxy-N,N-di-*n*-propyl-2-aminotetralin

4.4 Antipsychotic Drugs

The discovery of dopamine receptors was due to research into the mechanism of antipsychotic drugs (Seeman & Van Tol, 1994). It was shown using active and inactive enantiomers (eg + & - butaclamol), that neuroleptics (antipsychotics) specifically blocked dopamine receptors (Seeman *et al*, 1975). Now that these receptors have been cloned, this is facilitating the discovery of selective drugs for the treatment of diseases of the motor and cognition systems such as Parkinson's disease (dopaminergic degeneration), Huntington's chorea, and schizophrenia (type I, due to dopaminergic overactivity) (Seeman, 1987).

All neuroleptics except clozapine have been shown to occupy >60% of D_2 receptors under therapeutic conditions, however clozapine occupies only 20% (Karbe, 1991). Clozapine is an antipsychotic drug used in the treatment of type I schizophrenia, proposed to involve overactivity in dopaminergic systems in the CNS. Evidence for this includes the increase in density of the D_2 and D_3 receptors by 10% and D_4 by 600% (Seeman *et al,* 1993). Clozapine is classed as an atypical antipsychotic because it doesn't cause the Parkinson's-like side effects, which appear with other antipsychotic drug treatments. This is thought to be because the D_4 receptor is present at low levels in the basal ganglia (where the degeneration of the dopaminergic systems of Parkinson's sufferers occurs), and higher levels in the limbic system associated with the dopaminergic system overaction, (nigrostriatal, mesolimbic and mesocortical dopaminergic pathways are involved) (Watson & Arkinstall, 1994).

4.5 Aims of Research

This section of work arose from the need to persue a line of research with guarenteed results within the time-span of an MSc project. Although section A involved an interesting and valid area of research it was covering new ground and may not have produced satisfactory results within the given time. Therefore, it was decided, along with my CASE sponsors 'Syntex Ltd.' that the area of research should be altered slightly.

As they had a particular interest in the D_2-like dopamine receptors it was decided that this should be the area of focus.

The aim was to clone two of the D_2-like dopamine receptors, then to express them and perform ligand-binding assays, to build up a pharmacological profile. It was then hoped that chimera between the two could be created (as mentioned in section

A, 1.9) to investigate the molecular basis of the differential pharmacology of receptor type. Ideally D_2 and D_3 receptors would be used as these share the most homology, differing at very few residues for the same species and are therefore more suitable for chimera design.

SECTION B

CHAPTER 5

CLONING OF MOUSE $D2_S$ RECEPTOR

52Cloning of the short-form mouse D_2 receptor

5.1 Background

Rodents, particularly rats and mice are frequently used in molecular experimentation, both for general research into molecular biology and also for specific species research to discover their unique biology and the similarities and differences in relation to other species. This has lead to a spectrum of similarities to humans being discovered and utilised, particularly in the field of molecular biology. They are utilised as models for the human systems with which they share homology and the results have become more applicable to humans as strains have been developed with certain physical and genetic characteristics to facilitate their use. Ethically, some of the most useful molecular genetic experiments such as transgenic work can not be performed on human subjects, the use of laboratory animal such as mice allows research in this ground breaking area.

Mouse and rat receptor genes share a relatively high homology with the human sequences (Figure 5.11, p52a), and as mouse brain tissue is readily available in the laboratory, it was decided to isolate and study the D_2 dopamine receptor from mouse striatal tissue, where this receptor is relatively abundant. At the time of this work only the long form (D_{2L}) of this mouse receptor was available from the GenEMBL database. So sequence from both ends of the ORF (open reading frame) was used to design PCR primers as the short isoform of the receptor is a splice variant and therefore will also be amplified by the same primers.

The total length of the mouse D_2 long isoform is 1419bp (444 aa) and the short form is 1332bp (415 aa) with a 87bp (29 aa) fragment being spliced from the long isoform between 723bp and 811bp at the RNA level.

Figure 5.11 Alignment of the mouse, rat and human dopamine D_2 receptor peptide sequences (long isoforms), showing the high level of homology. Differences in sequence are indicated in bold type.

```
          1                                                   50
mouse     taaggaavrg cggswkpraa gafsgpgrpm a*ravppsgp tapMDPLNLS
  rat     .aaggaavrg cggswkppaa gafsgpgrhm a*ravppsgp tapMDPLNLS
human     .......... .......... .......... ....wppsrs talMDPLNLS

         51                                                  100
mouse     WYDDDLERQN WSRPFNGSEG KADRPHYNYY AMLLTLLIFI IVFGNVLVCM
  rat     WYDDDLERQN WSRPFNGSEG KADRPHYNYY AMLLTLLIFI IVFGNVLVCM
human     WYDDDLERQN WSRPFNGSDG KADRPHYNYY ATLLTLLIAV IVFGNVLVCM

         101                                                 150
mouse     AVSREKALQT TTNYLIVSLA VADLLVATLV MPWVVYLEVV GEWKFSRIHC
  rat     AVSREKALQT TTNYLIVSLA VADLLVATLV MPWVVYLEVV GDWKFSRIHC
human     AVSREKALQT TTNYLIVSLA VADLLVATLV MPWVVYLEVV GEWKFSRIHC

         151                                                 200
mouse     DIFVTLDVMM CTASILNLCA ISIDRYTAVA MPMLYNTRYS SKRRVTVMIA
  rat     DIFVTLDVMM CTASILNLCA ISIDRYTAVA MPMLYNTRYS SKRRVTVMIA
human     DIFVTLDVMM CTASILNLCA ISIDRYTAVA MPMLYNTRYS SKRRVTVMIS

         201                                                 250
mouse     IVWVLSFTIS CPLLFGLNNT DQNECIIANP AFVVYSSIVS FYVPFIVTLL
  rat     IVWVLSFTIS CPLLFRLNNT DQGECIIANP AFVVYSSIVS FYVPFIVTLL
human     IVWVLSFTIS CPLLFGLNNA DQNECIIANP AFVVYSSIVS FYVPFIVTLL

         251                                                 300
mouse     VYIKIYIVLR KRRKRVNTKR SSRAFRANLK TPLKGNCTHP EDMKLCTVIM
  rat     VYIKIYIVLR KRRKRVNTKR SSRAFRANLK TPLKGNCTHP EDMKLCTVIM
human     VYIKIYIVLR RRRKRVNTKR SSRAFRAHLR APLKGNCTHP EDMKLCTVIM

         301                                                 350
mouse     KSNGSFPVNR RRMDAARRAQ ELEMEMLSST SPPERTRYSP IPPSHHQLTL
  rat     KSNGSFPVNR RRMDAARRAQ ELEMEMLSST SPPERTRYSP IPPSHHQLTL
human     KSNGSFPVNR RRVEAARRAQ ELEMEMLSST SPPERTRYSP IPPSHHQLTL

         351                                                 400
mouse     PDPSHHGLHS NPDSPAKPEK NGHAKIVNPR IAKFFEIQTM PNGKTRTSLK
  rat     PDPSHHGLHS NPDSPAKPEK NGHAKIVNPR IAKFFEIQTM PNGKTRTSLK
human     PDPSHHGLHS TPDSPAKPEK NGHAK.DHPKIAKIFEIQTM PNGKTRTSLK

         401                                                 450
mouse     TMSRRKLSQQ KEKKATQMLA IVLGVFIICW LPFFITHILN IHCDCNIPPV
  rat     TMSRRKLSQQ KEKKATQMLA IVLGVFIICW LPFFITHILN IHCDCNIPPV
human     TMSRRKLSQQ KEKKATQMLA IVLGVFIICW LPFFITHILN IHCDCNIPPV

         451
mouse     LYSAFTWLGY VNSAVNPIIY TTFNIEFRKA FMKILHC*
  rat     LYSAFTWLGY VNSAVNPIIY TTFNIEFRKA FMKILHC*
human     LYSAFTWLGY VNSAVNPIIY TTFNIEFRKA FLKILHC*
```

5.2 Experimental Design

5.21 Overview

Total RNA was isolated from mouse striatal tissue and first strand cDNA was synthesised from this. The cDNA was then used as a template for nested PCR. The initial round of PCR was performed using a pair of primers designed using the sequences immediately 5' and 3' of the D_{2L} receptor open-reading frame (ORF) sequence (GenEMBL x55674). Then a second round of PCR was performed on the first round product using a second, inner pair of primers designed to encompass the 5' and 3' ends of the D_{2L} receptor ORF sequence, with restriction enzyme sites incorporated at the primers' 5' ends.

Products of the expected size from the second round of PCR, were purified and cloned into the T-vector 'pT7Blue(R)' (Novagen) where the identity of each clone was determined initially by restriction and those which appeared correct sequenced using United States Biochemical 'Sequenase 2.0' (T7 DNA Polymerase). When a D_2 clone was confirmed it was subcloned into 'pcDNA3' a mammalian expression vector (Invitrogen) and its orientation confirmed by restriction digest.

5.22 Polymerase Chain Reaction (PCR)

Nested primers were designed involving two separate pairs of primers. Pair A corresponded to sequence outside of the open reading frame (ORF) of the D_{2L} sequence (GenEMBL), while pair B corresponded to sequence at either end of the ORF (see Figures 5.221 & 5.222, p52a & 53a).

Figure 5.221 Mouse D_{2L} dopamine receptor nucleotide (GenBank X55674, Montmayeur*et al*, 1991) and amino acid sequence showing primer sites, the area spliced from the short isoform and restriction sites utilised for identification of the clone.

```
                 B
                 a                                  aagagccgtgccacccagtggcccc
                 m                                        A1
                 H
                 I
     actgccccaATGGATCCACTGAACCTGTCCTGGTACGATGATGATCTGGAGAGGCAGAAC
      B1          M  D  P  L  N  L  S  W  Y  D  D  D  L  E  R  Q  N

  52 TGGAGCCGGCCCTTCAATGGGTCCGAAGGGAAGGCAGACAGGCCCCACTACAACTACTAT
  18 W  S  R  P  F  N  G  S  E  G  K  A  D  R  P  H  Y  N  Y  Y

 112 GCCATGCTGCTCACCCTCCTCATCTTTATCATCGTCTTTGGCAATGTGCTGGTGTGCATG
  38 A  M  L  L  T  L  L  I  F  I  I  V  F  G  N  V  L  V  C  M

 172 GCTGTATCACGAGAGAAGGCTTTGCAGACCACCACCAACTACCTGATAGTCAGCCTCGCT
  58 A  V  S  R  E  K  A  L  Q  T  T  T  N  Y  L  I  V  S  L  A

 232 GTGGCCGATCTTCTGGTGGCCACACTGGTTATGCCCTGGGTCGTCTATCTGGAGGTGGTG
  78 V  A  D  L  L  V  A  T  L  V  M  P  W  V  V  Y  L  E  V  V

 292 GGTGAGTGGAAATTCAGCAGGATTCACTGTGACATCTTTGTCACTCTGGATGTCATGATG
  98  G  E  W  K  F  S  R  I  H  C  D  I  F  V  T  L  D  V  M  M

                                                              P
                                                              v
                                                              u
 352 TGCACAGCAAGCATCTTGAACCTGTGTGCCATCAGCATCGACAGGTACACAGCTGTGGCC
 118 C  T  A  S  I  L  N  L  C  A  I  S  I  D  R  Y  T  A  V  A

 412 ATGCCTATGTTGTATAACACACGCTACAGCTCCAAGCGCCGAGTTACTGTCATGATCGCC
 138 M  P  M  L  Y  N  T  R  Y  S  S  K  R  R  V  T  V  M  I  A

 472 ATTGTCTGGGTCCTGTCCTTCACCATCTCTTGCCCACTGCTCTTTGGACTCAACAACACA
 158 I  V  W  V  L  S  F  T  I  S  C  P  L  L  F  G  L  N  N  T

 532 GACCAGAATGAGTGTATCATTGCCAACCCTGCCTTCGTGGTCTACTCCTCCATCGTCTCG
 178 D  Q  N  E  C  I  I  A  N  P  A  F  V  V  Y  S  S  I  V  S

 592 TTCTACGTGCCCTTCATCGTCACCCTGCTGGTCTATATCAAAATCTACATCGTTCTCCGC
 198 F  Y  V  P  F  I  V  T  L  L  V  Y  I  K  I  Y  I  V  L  R

 652 AAGCGTCGGAAGCGGGTCAACACCAAGCGTAGCAGCCGAGCTTTCAGAGCCAACCTGAAG
 218 K  R  R  K  R  V  N  T  K  R  S  S  R  A  F  R  A  N  L  K
```

```
712 ACACCACTCAAGGGCAACTGTACCCACCCTGAGGACATGAAACTCTGCACCGTTATCATG
238 T  P  L  K  G  N  C  T  H  P  E  D  M  K  L  C  T  V  I  M

                                                              S
                                                              a
                                                              c
772 AAGTCTAATGGGAGTTTCCCAGTGAACAGGCGGAGAATGGATGCTGCCCGCCGAGCTCAG
258 K  S  N  G  S  F  P  V  N  R  R  R  M  D  A  A  R  R  A  Q

832 GAGCTGGAAATGGAGATGCTGTCAAGCACCAGCCCCCCAGAGAGGACCCGGTATAGCCCC
278 E  L  E  M  E  M  L  S  S  T  S  P  P  E  R  T  R  Y  S  P

892 ATCCCTCCCAGTCACCACCAGCTCACTCTCCCCGATCCATCCCACCACGGTCTACATAGC
298 I  P  P  S  H  H  Q  L  T  L  P  D  P  S  H  H  G  L  H  S

952 AACCCTGACAGTCCTGCCAAACCAGAAAAGAATGGGCATGCCAAGATTGTCAATCCCAGG
318 N  P  D  S  P  A  K  P  E  K  N  G  H  A  K  I  V  N  P  R

1012 ATTGCCAAGTTCTTTGAGATCCAGACCATGCCCAATGGCAAAACCCGGACCTCCCTTAAG
 338 I  A  K  F  F  E  I  Q  T  M  P  N  G  K  T  R  T  S  L  K

1072 ACGATGAGCCGCAGGAAGCTCTCCCAGCAGAAGGAGAAGAAAGCCACTCAGATGCTTGCC
 358 T  M  S  R  R  K  L  S  Q  Q  K  E  K  K  A  T  Q  M  L  A

1132 ATTGTTCTTGGTGTGTTCATCATCTGCTGGCTGCCCTTCTTCATCACGCACATCCTGAAT
 378 I  V  L  G  V  F  I  I  C  W  L  P  F  F  I  T  H  I  L  N

1192 ATACACTGTGACTGCAACATCCCACCAGTCCTCTACAGCGCCTTCACATGGCTGGGCTAT
 398 I  H  C  D  C  N  I  P  P  V  L  Y  S  A  F  T  W  L  G  Y

1252 GTCAACAGTGCCGTGAACCCCATCATCTATACCACCTTCAACATTGAGTTCCGCAAGGCC
 418 V  N  S  A  V  N  P  I  I  Y  T  T  F  N  I  E  F  R  K  A

1312 TTCATGAAGATCCTGCACTGCtgagtctgcccttgcctgcacagcagctgcttgccgcc
 438 F  M  K  I  L  H  C  *                                    A2
             B2

     tccctgcctaggcag
```

The PCR primer sites are double underlined and labelled A1/A2 and B1/B2. The 87bp region underlined from 723bp shows the region spliced to form the short isoform. The five underlined 20mers show the position of sequencing primers used to sequnce the clone in both directions over its entire length.

Enzymes that cut - BamHI @ 3 bp
PvuII @ 404 bp
SacI @ 828 bp
(EcoRI does not cut)

ORF length - 1332 bp = 444 aa

Figure 5.222 *Schematic diagram showing position of nested primers for amplification of $D2_S$*

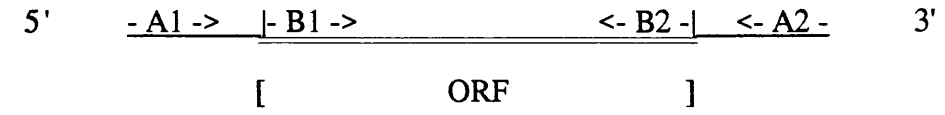


Table 5.223 Sequence of nested D2 receptor PCR primers

primer	Primer Nucleotide Sequence 5' 3'	Length (bp)	Tm (°C)	Sense
A1	AGAGCCGTGCCACCCAGTGG	22	68	Sense
A2	CCTAGGCAGGGAGGCGGCAA	22	68	Anti
B1	CGC**G'AATTC**TGCCCCAATGGATCCACTGAA	30	64	Sense
B2	GCG**G'AATTC**GCAGTGCAGGATCTTCATGAAG	30	66	Anti

Table 5.224 PCR reaction conditions

	Primary PCR	Secondary PCR	Duration
Hot-start	94°C	94°C	1 min
Denaturation	94°C	94°C	45 sec
Annealing x30	57°C	55°C	1 min
Extension	72°C	72°C	2 min
Soak	4°C	4°C	indefinite

Figure 5.221 shows the exact position of primer annealing to the dopamine D_2 receptor sequence and this is illustrated schematically in Figure 5.222. The actual primer sequences are displayed in Table 5.223 (p54) where it can be seen that the outer pair (A1 & A2) are totally homologous to sequences 5' & 3' of the mouse D2 dopamine receptor. Whereas the inner primers (B1 & B2) have total homology to the sequence at the 3' end but have non-homologous 5' ends which include EcoRI restriction sites to aid cloning.

The design of the primers took into account several basic factors in order to optimise their action. Because each pair was totally matched to the D_2 receptor sequence over at least 15 nucleotides to increase the probability of unique homologous priming, a non-homologous 5' end could be added to the inner primers without affecting their priming ability. The T_ms (melting temperatures) were balanced within a pair so that both primers annealed at around the same temperature. The 3' ends were designed to be non-homologous to each other to prevent primer-dimer formation and attempts were made to keep the G/C content to below 60% to avoid problems with secondary structures.

The reaction conditions were also designed to help prevent mis-priming by using hot-start PCR and short ramping times and the nested nature of the primers significantly increased the sensitivity of the reaction (McPherson et al, 1990). The reaction components and concentrations are shown in Methods (Chapter 8).

5.23 Cloning and Sequencing

PCR products from the second round of thermal cycling were run on a 1% agarose gel and the DNA collected using NA45 paper (Schleider & Schuell) as described in Chapter 8.

The pT7Blue(R) T-vector (R & D Systems) was used to clone the products, as it is specifically designed for the cloning of DNA fragments produced by PCR. Taq DNA polymerase leaves single 3' A-nucleotide overhangs on the reaction products (Clark, 1988). The vector has been digested with *EcoR1* and single 3' dT residues added at each end, allowing direct ligation of the PCR product, but preventing self-ligation.

After ligation and transformation (Methods, Chapter 8) individual clones were selected and restricted using the enzymes *BamHI, SacI, PvuII* and *EcoRI* which permit positive identification of a dopamine D_2 receptor clone and determine its orientation within the vector.

5.3 Results

5.31 Polymerase Chain Reaction

The results of the PCR reaction are displayed in Figure 5.311 (p58). Reactions using cortex cDNA as a template were unsuccessful, however striatal cDNA produced four definite bands at approximately 1.3, 0.7, 0.4 and 0.3kb. The expected length of the long D_2 isoform is 1332bp and the short form 1245bp, therefore the largest band (which also had the strongest intensity) was collected.

5.32 Cloning and restriction

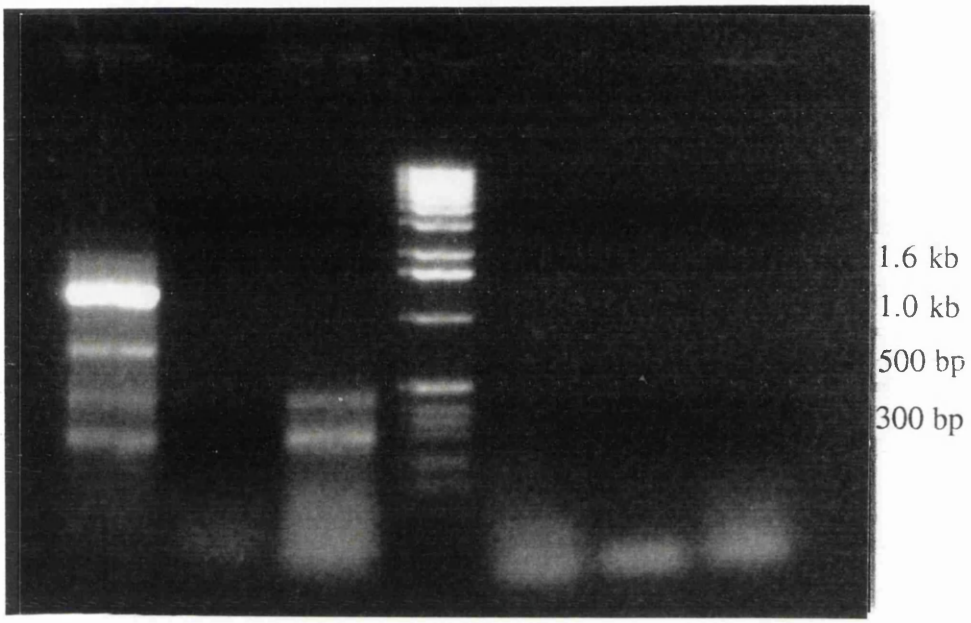
Figure 5.321 (p59) is a schematic diagram of the T-vector pT7Blue(R) containing the desired D_2 receptor clone. It illustrates how the restriction enzymes previously mentioned can be used to identify correct inserts.

Table 5.322 Restriction fragment sizes produced by digesting either D_2 receptor isoform cloned into pT7Blue(R) with different enzymes

	Positive Orientation (bp)		Negative Orientation (bp)	
	D_{2S}	D_{2L}	D_{2S}	D_{2L}
EcoRI	1258 2899	1345 2899	1258 2899	1345 2899
BamHI	1251 2906	1338 2906	23 4134	23 4221
SacI	449 3708	536 3708	780 3377	867 3377
PvuII	967 581 2609	1054 581 2609	1011 537 2609	1098 537 2609

The above table predicts the approximate size of fragments obtained from digestion of mouse D_2 dopamine receptors in pT7Blue. Figure 5.323 (p60) shows the restriction digest result from a clone which appears to contain a D_2 receptor in the correct orientation. This clone was selected and sequenced.

Figure 5.311 Product of second round PCR using the nested dopamine D_{2S} receptor primer pairs with mouse striatal cDNA template.



Key to lanes

1 Second round PCR of striatal cDNA using nested D_2 primers
2 First round PCR of striatal cDNA using outer D_2 primers
3 Same as lane 1 after collection of larger band sizes using NA45 paper
4 250ng 1kb marker (Gibco BRL)
5 Second round PCR of cortex cDNA using nested D_2 primers
6 Zero template
7 First round PCR of cortex cDNA using outer D_2 primers

PCR Programme

94°C - 1 min
⇓
94°C - 45 sec
57°C - 1 min x 30 cycles
72°C - 2 min
⇓
4°C soak

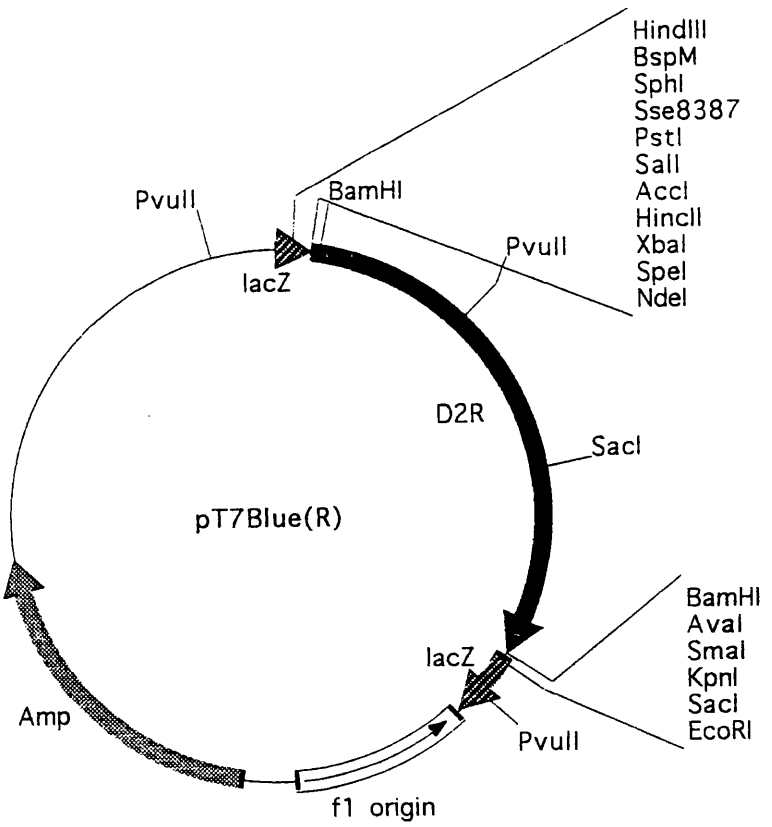
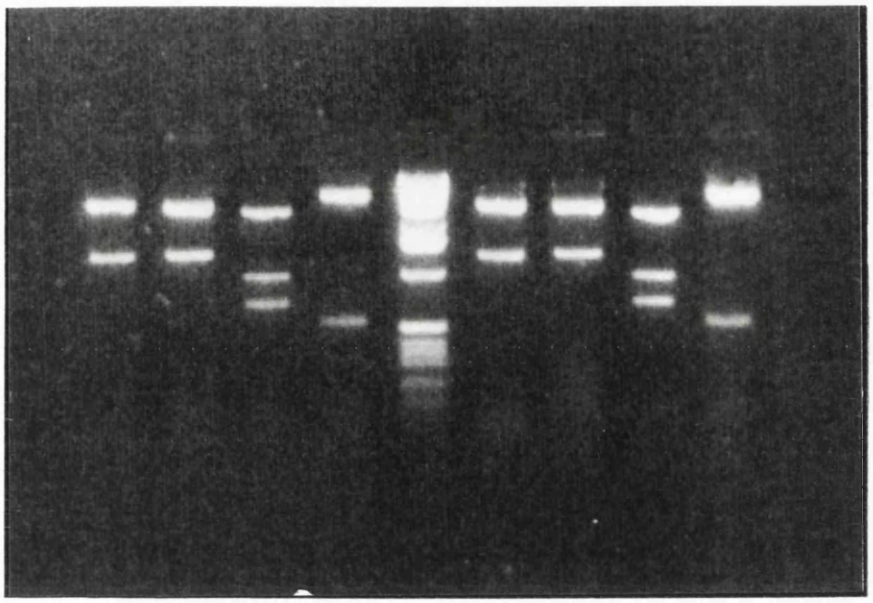


Figure 5.321 Mouse D_{2S} (short isoform) dopamine receptor ligated into pT7Blue(R) T-cloning vector, 4.2kb (R & D Systems).

From the map it can be seen how the use of selected restriction enzymes (*BamHI, SacI, PvuII and EcoRI*) can be used to confirm the insert and to determine its orientation within the vector.

Figure 5.323 Identification of a D_2 receptor clone in the plasmid vector pT7 (R & D Systems) by restriction fragment length polymorphism.



Key to lanes		
	1 & 6	*Eco RI* digestion
	2 & 7	*Bam HI* digestion
	3 & 8	*Pvu II* digestion
	4 & 9	*Sac I* digestion
	5	250ng 1kb marker (Gibco BRL)

Products were electrophoresed on a 1% agarose gel at 50 volts for 1 hour

Product sizes			
	1	≈	3000 & 1300 bp
	2	≈	3000 & 1300 bp
	3	≈	2500, 1000 & 600 bp
	4	≈	3800 & 550 bp

Figure 5.331 Alignment of mouse D_{2S} dopamine receptor short isoform sequence[1], determined by dideoxy DNA sequencing, to published mouse D_{2L} receptor[2] (x55674, Montmayeur *et al*, '91)

```
1ctgccccaATGGATCCACTGAACCTGTCCTGGTACGATGATGATCTGGAGAGGCAGAAC  51
2ctgccccaATGGATCCACTGAACCTGTCCTGGTACGATGATGATCTGGAGAGGCAGAAC

TGGAGCCGGCCCTTCAATGGGTCCGAAGGGAAGGCAGACAGGCCCCACTACAACTACTAT 111
TGGAGCCGGCCCTTCAATGGGTCCGAAGGGAAGGCAGACAGGCCCCACTACAACTACTAT

GCCATGCTGCTCACCCTCCTCATCTTTATCATCGTCTTTGGCAATGTGCTGGTGTGCATG 171
GCCATGCTGCTCACCCTCCTCATCTTTATCATCGTCTTTGGCAATGTGCTGGTGTGCATG

GCTGTATCACGAGAGAAGGCTTTGCAGACCACCACCAACTACCTGATAGTCAGCCTCGCT 231
GCTGTATCACGAGAGAAGGCTTTGCAGACCACCACCAACTACCTGATAGTCAGCCTCGCT

GTGGCCGATCTTCTGGTGGCCACACTGGTTATGCCCTGGGTCGTCTATCTGGAGGTGGTG 291
GTGGCCGATCTTCTGGTGGCCACACTGGTTATGCCCTGGGTCGTCTATCTGGAGGTGGTG

GGTGAGTGGAAATTCAGCAGGATTCACTGTGACATCTTTGTCACTCTGGATGTCATGATG 351
GGTGAGTGGAAATTCAGCAGGATTCACTGTGACATCTTTGTCACTCTGGATGTCATGATG

TGCACAGCAAGCATCTTGAACCTGTGTGCCATCAGCATCGACAGGTACACAGCTGTGGCC 411
TGCACAGCAAGCATCTTGAACCTGTGTGCCATCAGCATCGACAGGTACACAGCTGTGGCC

ATGCCTATGTTGTATAACACACGCTACAGCTCCAAGCGCCGAGTTACTGTCATGATCGCC 471
ATGCCTATGTTGTATAACACACGCTACAGCTCCAAGCGCCGAGTTACTGTCATGATCGCC

ATTGTCTGGGTCCTGTCCTTCACCATCTCTTGCCCACTGCTCTTTGGACTCAACAACACA 531
ATTGTCTGGGTCCTGTCCTTCACCATCTCTTGCCCACTGCTCTTTGGACTCAACAACACA

GACCAGAATGAGTGTATCATTGCCAACCCTGCCTTCGTGGTCTACTCCTCCATCGTCTCG 591
GACCAGAATGAGTGTATCATTGCCAACCCTGCCTTCGTGGTCTACTCCTCCATCGTCTCG

TTCTACGTGCCCTTCATCGTCACCCTGCTGGTCTATATCAAAATCTACATCGTTCTCCGC 651
TTCTACGTGCCCTTCATCGTCACCCTGCTGGTCTATATCAAAATCTACATCGTTCTCCGC

AAGCGTCGGAAGCGGGTCAACACCAAGCGTAGCAGCCGAGCTTTCAGAGCCAACCTGAAG 711
AAGCGTCGGAAGCGGGTCAACACCAAGCGTAGCAGCCGAGCTTTCAGAGCCAACCTGAAG

ACACCACTCAAG..................................................  723
ACACCACTCAAGGGCAACTGTACCCACCCTGAGGACATGAAACTCTGCACCGTTATCATG 771

.....................................GATGCTGCCCGCCGAGCTCAG 744
AAGTCTAATGGGAGTTTCCCAGTGAACAGGCGGAGAATGGATGCTGCCCGCCGAGCTCAG 831

GAGCTGGAAATGGAGATGCTGTCAAGCACCAGCCCCCCAGAGAGGACCCGGTATAGCCCC 804
GAGCTGGAAATGGAGATGCTGTCAAGCACCAGCCCCCCAGAGAGGACCCGGTATAGCCCC 891
```

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ATCCCTCCCAGTCACCACCAGCTCACTCTCCCCGATCCATCCCACCACGGTCTACATAGC 864
ATCCCTCCCAGTCACCACCAGCTCACTCTCCCCGATCCATCCCACCACGGTCTACATAGC 951


AACCCTGACAGTCCTGCCAAACCAGAAAAGAATGGGCATGCCAAGATTGTCAATCCCAGG 924
AACCCTGACAGTCCTGCCAAACCAGAAAAGAATGGGCATGCCAAGATTGTCAATCCCAGG 1011

ATTGCCAAGTTCTTTGAGATCCAGACCATGCCCAATGGCAAAACCCGGACCTCCCTTAAG 984
ATTGCCAAGTTCTTTGAGATCCAGACCATGCCCAATGGCAAAACCCGGACCTCCCTTAAG 1071


ACGATGAGCCGCAGGAAGCTCTCCCAGCAGAAGGAGAAGAAAGCCACTCAGATGCTTGCC 1044
ACGATGAGCCGCAGGAAGCTCTCCCAGCAGAAGGAGAAGAAAGCCACTCAGATGCTTGCC 1131


ATTGTTCTTGGTGTGTTCATCATCTGCTGGCTGCCCTTCTTCATCACGCACATCCTGAAT 1104
ATTGTTCTTGGTGTGTTCATCATCTGCTGGCTGCCCTTCTTCATCACGCACATCCTGAAT 1191


ATACACTGTGACTGCAACATCCCACCAGTCCTCTACAGCGCCTTCACATGGCTGGGCTAT 1164
ATACACTGTGACTGCAACATCCCACCAGTCCTCTACAGCGCCTTCACATGGCTGGGCTAT 1251


GTCAACAGTGCCGTGAACCCCATCATCTATACCACCTTCAACATTGAGTTCCGCAAGGCC 1224
GTCAACAGTGCCGTGAACCCCATCATCTATACCACCTTCAACATTGAGTTCCGCAAGGCC 1311


TTCATGAAGATCCTGCACTGCt 1245
TTCATGAAGATCCTGCACTGCt 1332
```

Short form ORF length - 1245 bp - 87 bp shorter than D_{2L} (1332bp)

The sequences are totally homologous over their coding regions, indicating that the PCR derived clone of D_{2S} does not contain any *Taq* introduced errors. The region spliced in the short form can clearly be seen from 723 - 810bp, this does not interrupt the ORF (Figure 5.221, p53a/b)

The frequency of transformation was low and the chromogenic selection (using IPTG and X-Gal), failed to indicate the presence of any clones containing inserts (ie a white colony). However, restriction digestion of one of the apparently 'negative' clones showed that it did in fact contain an insert of the correct size. This phenomenon could be due to the production of a fusion protein containing both the clone and a functional β-galactosidase protein, or possibly another start-site within β-galactosidase downstream from the insert site.

5.33 Sequencing

This positive clone was sequenced using the dideoxy method (Chapter 8). The T7 5' primer and U-19 3' primer were used to obtain the terminal sequences of the clone, then the inner PCR primers (B1 & B2) were used to sequence further into the clone. To obtain the central sequence five pairs of internal primers were synthesised so that the entire length of the clone could be sequenced in both directions (Figure 5.221, p53a/b). The resulting sequence (Figure 5.331,p61) shows that the short isoform of the mouse D_2 dopamine receptor has been cloned.

5.34 Subcloning into pcDNA3 (Invitrogen)

The EcoRI sites incorporated into the inner PCR primers were used to subclone the receptor into an expression vector, pcDNA3 (Invitrogen). This vector contains various features including the CMV (cytomegalovirus) promoter for high-level expression, the bovine growth hormone polyadenylation signal for polyadenylation of transcribed mRNAs, neomycin resistance for G418 selection and an EcoRI site in the polylinker. The subclone could insert in either orientation and therefore restriction enzymes were again used to orientate resulting clones. Figure 5.341 illustrates how *PvuII* was used to select clones in the positive orientation ready for expression in rat-1 fibroblasts.

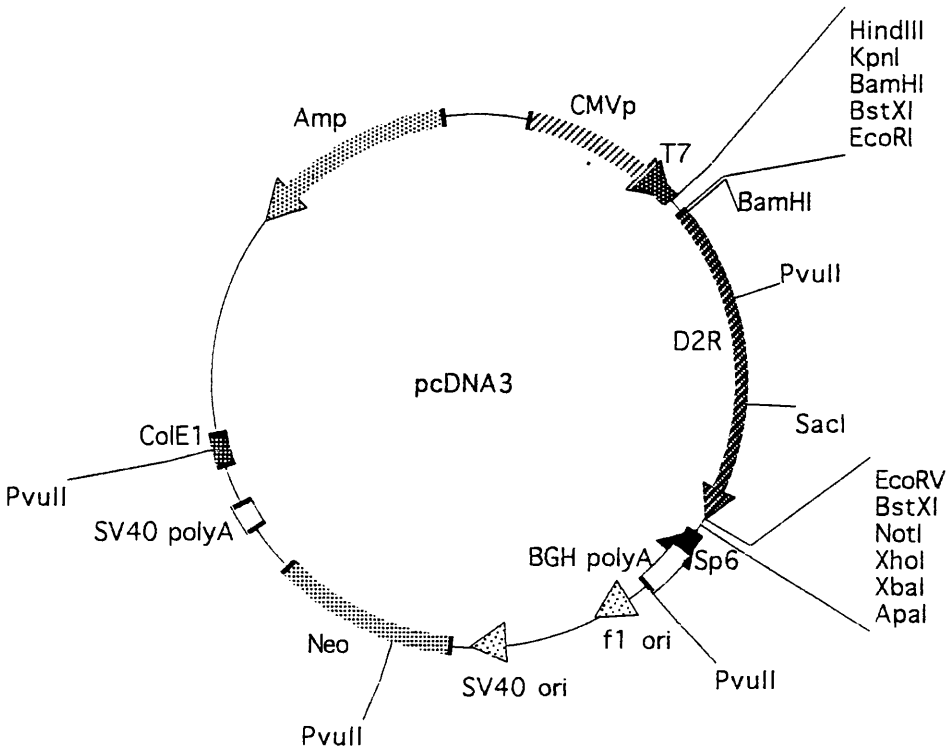


Figure 5.341 Mouse D_{2S} (short isoform) dopamine receptor ligated into pcDNA3 mammalian expression vector, 5.6kb (Invitrogen), using *EcoRI* sites engineered into the PCR primer 5' ends.

From the map it can be seen that restriction with the enzyme PvuII allows orientation of clones so that the ORF reads forward.

Correct orientation	Incorrect orientation
3284 bp	3944 bp
1410 bp	1097 bp
1097 bp	1069 bp
1069 bp	750 bp

5.4 Discussion

The use of cortex cDNA template was unsuccessful which was probably due to D_2 receptors being expressed at a lower level in this region. It may also be that the cortex template is of a lower quality as when it was used with control primers for the α-subunit of the $GABA_A$ receptor (Chapter 2), the amount of product seen when using the cortex template was less than that produced from striatal template at the same conditions (Figure 2.62).

It was also suprising that more copies of the D_2 dopamine receptor were not cloned as it had obviously amplified successfully. The transformation frequency was consistently low and of the few clones which were isolated only one gave the correct digest pattern. The situation was also complicated by the failure of blue white selection as the eventual positive clone had a blue phenotype which was probably caused by the fragment inserting in frame and causing a *lacZ* fusion protein or transcription restart.

It is clear that the dopamine D_{2S} receptor has been successfully cloned and can now be expressed in order to study its pharmacology.

SECTION B

CHAPTER 6

PHARMACOLOGY

Pharmacological Investigation of the Mouse D2S (Short Form) Dopamine Receptor

The cloned D_{2S} (short-isoform) dopamine receptor from mouse striatum was investigated for its ligand binding properties when expressed in rat-1 fibroblast cells. The same binding assays were also carried out on rat striatal tissue containing both D_{2S} and D_{2L} receptors as a comparison of pharmacological profiles.

A rapid membrane filtration technique was employed, along with a specific radioligand. This is a relatively simple method for investigating direct binding of compounds to receptors and is therefore frequently used for many receptor based systems such as neurotransmittion and hormone action.

6.1 Saturation Assay

This assay determines the amount of a radioligand specifically bound to a receptor species for varying concentrations of the ligand. From the graphical interpretation of this data the total number of binding sites (B_{max}) can be calculated and also the ligand's dissociation constant value (K_D). K_D is defined as the concentration of radioligand at which 50% of the total number of receptors are labelled and is indicative of the affinity of the ligand for the receptor. A known quantity of protein (ie membrane homogenate) was incubated with a range of different radioligand concentrations. Disintegration per minute (dpm) of the tritium isotope was measured using a scintillation counter. Using the specific activity of the radioligand the recorded counts could be converted to radioligand concentrations using equation 1, therefore allowing quantitative measurement of the ligand by its radioactive nature.

$$1\ Ci = 2.197 \times 10^{12}\ dpm \qquad \textit{(equation 1)}$$

A commercially available tritiated ligand was used, [^{3}H]-spiperone (95 Ci/mmol) (DuPont) which is a selective dopamine D_2 receptor antagonist . It is ideal as it appears to interact with the receptor in the same way as the untritiated form and has a long half-life of 12 years.

A saturation assay is composed of several assay forms measuring total and non-specific binding so that the specific binding of [^{3}H]-spiperone to D_2 receptors can be calculated;

a) Total binding assay

This is filtered after incubation in order to collect counts representing total binding of radioligand to both specific (D_2 receptors) and non-specific sites, over the range of radioligand concentrations used.

b) Non-specific binding assay (NSB)

These tubes include the drug (+)butaclamol at 10^{-6} M. This is a D_2 receptor specific ligand at a high concentration (100 fold excess) in order to ensure that it occupies all of the D_2 receptor sites present preferentially over [^{3}H]-spiperone. Therefore any radioligand which remains after filtration can be assumed to be non-specifically bound to the membrane or filter.

3) Unfiltered assay

This is identical to the total assay but remains unfiltered after incubation. Aliquots are counted to determine the concentration of radioligand used in the assay.

Specific binding of the radioligand to the receptor under investigation (D_2) is calculated by subtraction of the counts of the NSB assay from that of the total

$$B_{max} = R_1 \text{ dpm} \times \frac{1}{2 \times 10^{12} \text{ dpm/Ci}} \times \frac{1}{95 \text{Ci(mmol}^{-1})}.$$

$$= \frac{R_1 \text{ mmol}}{190 \times 10^{12}}$$

$$= \frac{R_1}{190} \times 10^{-15} \text{ mol}$$

$$= \frac{R_1}{190} \text{ femtomoles}$$

binding, for each radioligand concentration. The amount of free ligand is calculated by subracting total bound [^{3}H] from the unfiltered assay (ie total [^{3}H]).

When the curve is plotted for the specific binding of the radioligand, against the free concentration (mol l^{-1}), for an increasing range of concentrations, binding is shown to increase until it reaches a steady state, indicated by a plateau on the curve. This is the saturation point, where all available receptor molecules are occupied by the radioligand, it is known as R_1, representing the concentration of [^{3}H]-spiperone (mol l^{-1}) required to saturate the specific binding sites (D_2 receptors). It is required to calculate the B_{max} using the following equation:

$$B_{max} = \frac{\frac{R_1}{2000}}{\text{mg protein per tube}} = \text{femtomoles receptor per mg protein} \qquad \textit{(equation 2)}$$

The experimental data was interpreted using the computer programme 'Ligand' (Munson & Rodbard, 1980), a non-linear least squares fitting programme. It determines 'best-fit' curves for the values obtained in the assays (see Figures 6.412 & 6.414, p71a), therefore facilitating calculation of K_D and B_{max} values.

The work of Scatchard (1949) on the graphical analysis of saturation data was developed by Rosenthal (1967), who showed that by plotting the specific bound ligand/free ligand against specific bound ligand a linear slope is produced. The negative reciprocal of the gradient produces the K_D value for the radioligand, whilst the intercept at x-axis gives the B_{max} value (Figures 6.413 & 6.415, p71b). The drug ketanserin was included in the assays at a final saturating assay concentration (F.A.C.) of 1 x 10^{-7}M. As [^{3}H]-spiperone also has affinity for $5HT_2$ receptors, the addition of ketanserin prevents it binding at these sites.
Even though steps were taken to reduce the D_2 non-specific binding of the radioligand, there was unavoidable binding to the membrane and filter which could

reduce the accuracy of the assay. However, this factor was removed by subtraction of NSB from total binding allowing calculation of the specific binding.

6.2 Competition Assay

This assay was used to study the relative affinities of various ligands for receptors. As with the saturation assay a standard quantity of protein was used, however the concentration of radioligand was also fixed and ranges of increasing concentrations of various unlabelled competitor ligands were included. The unlabelled ligand competes with the radioligand for the receptor binding sites and at increasing concentrations of unlabelled competitor the binding of the radioligand decreases, ie the competitor is inhibiting the radioligand binding. The ability of each competing compound to inhibit the specific binding of the radioligand can be quantitatively evaluated by determining its IC_{50} (inhibitory concentration) value. This is the concentration of competing ligand at which 50% of specific radioligand binding is inhibited. Therefore the greater the affinity of the ligand for the receptor the lower its IC_{50}. It can be calculated using a curve fitting programme, or the following equation:

$$\log \frac{B_i}{B_0 - B_i} = n \log[I] - n \log IC_{50} \qquad \textit{(equation 3)}$$

where: B_i = amount of bound radioligand in presence of competitor

B_0 = amount of bound radioligand in absence of competitor

I = competitor (inhibitor)

When the first half of this equation is plotted against log[I] the intercept on the x axis gives the IC_{50} value and the slope of the line, (the slope factor, n_H) indicates whether binding is simple or complex. An n_H value of one (unity) indicates binding to one site following mass action law, however a value > 1 may indicate

positive cooperativity and < 1 can be due to multiple receptor subtypes, receptor states of multiple step binding.

The IC_{50} could then be converted to the K_i (equilibrium dissociation constant) value for the ligand using equation 4, derived by Cheng & Prusoff in 1973.

$$\frac{IC_{50}}{1+\frac{[L]}{K_D}} = K_i \text{ (mol } l^{-1}\text{)}$$

[L] = ^{3}H-ligand conc.

(equation 4: Cheng-Prusoff equation)

The negative log of this (pK_i) provides an index which can be used to compare the affinities of different ligands for a receptor.

6.21 Receptor Activation State

Dopamine receptors, like G-protein linked receptors (GPLRs) in general, can occur in two main dynamic states. When in complex with a G protein the receptor has a high affinity for agonists, which is converted to a low affinity when dissociated from the G protein. In normal experimental conditions there are receptors both in the high and low affinity states and therefore a non-hydrolysable analogue of GTP (Gpp(NH)p) was incorporated into the assay. This causes dissociation of G proteins and the convertion of all receptors to the lower affinity state giving a more standard binding result and shifting the competition curve for that drug to the right, (ie K_i increases, pK_i decreases).

6.3 Protein Assay

The membrane and cell homogenates used in the binding assays were themselves assayed before use, using a Pierce BSA Assay Kit, to establish their protein concentration. This allowed standardisation of results from the saturation experiment, as the Bmax is corrected for assay protein concentration.

RESULTS

6.41 Saturation Binding of [^{3}H]-spiperone

Rat-1-fibroblast cells were stably transfected with the wild-type mouse D_{2S} dopamine receptor, binding assays were performed on membrane preparations from these and compared with D_2 receptor preparations from rat striatal tissue. The results from this assay show the binding of [^{3}H]-spiperone to the D_2 receptor population in rat stiatal tissue and to the cloned mouse D_{2S} expressed in rat-1 fibroblast cells. Protocols and conditions for the assays have been determined by extensive research at 'Syntex' (unpublished) and are similar to other published conditions for these assays (Guiramand *et al* 1995; Leysen *et al* 1992), (see 'Materials', Appendix I).

The results of the protein assay for the homogenates used in the saturation assays are shown in Table 6.411. The values were calculated using a standard curve generated using bovine serum albumin (BSA).

BSA Standard curve equation for striata homogenate $y = -2.84e^{-3} + 0.88x$

BSA Standard curve equation for cell homogenate $y = 9.273e^{-3} + 1.142x$

Table 6.411 Membrane Homogenate Protein Concentration (mg protein/tube)

Assay Number (n)	1	2	3
Striatal Homogenate	0.164	0.158	0.145
Clone Homogenate	0.149	0.149	0.149

The curves for the binding of the D_2 specific, displaceable radioligand [^{3}H]-spiperone (Figures 6.412 & 6.414, p71a), show that binding is saturable, and the corresponding Scatchard plots (Figures 6.413 & 6.415, p71b) indicate a one site

Figure 6.414 Saturation binding curve of [^{3}H]-spiperone binding in membrane preparations of rat-1 fibroblast cells expressing the mouse dopamine D_{2S} receptor

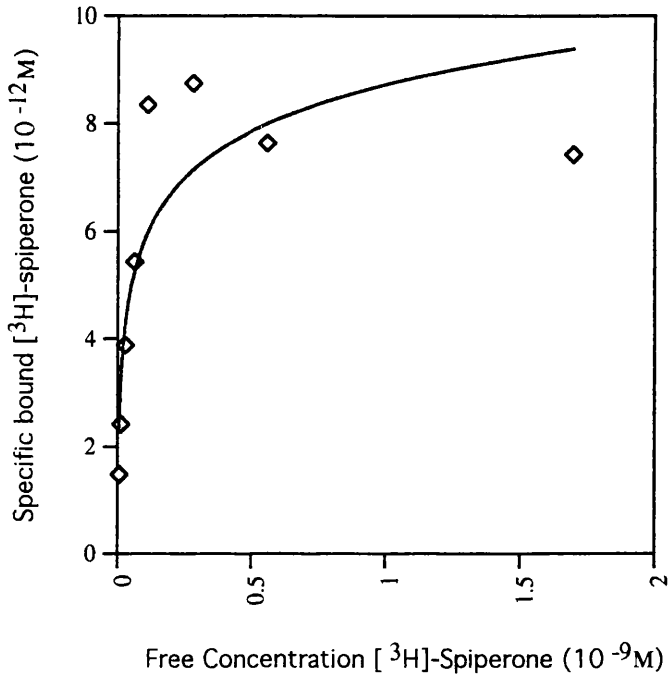


Figure 6.412 Saturation binding curve of [^{3}H]-spiperone binding D_2 dopamine receptors in rat striatal membrane preparation

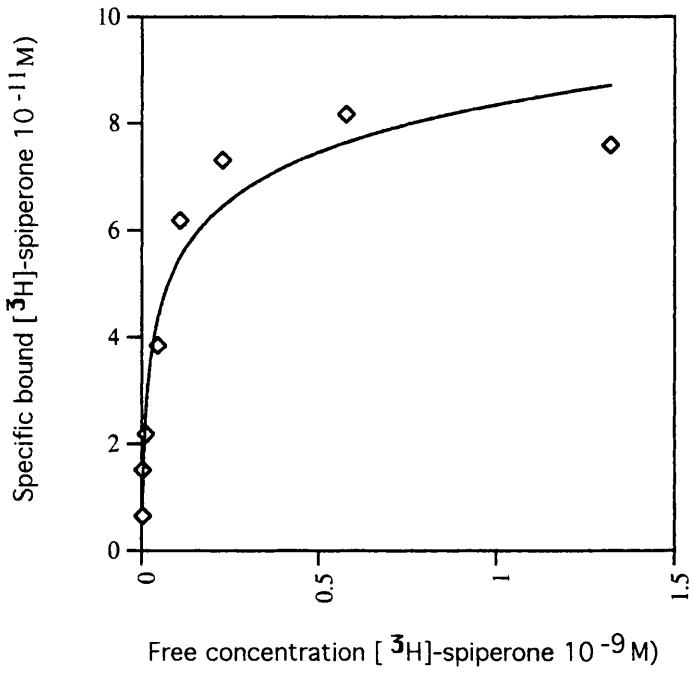


Figure 6.415 Scatchard plot of [^{3}H]-spiperone binding mouse dopamine D_{2S} receptor

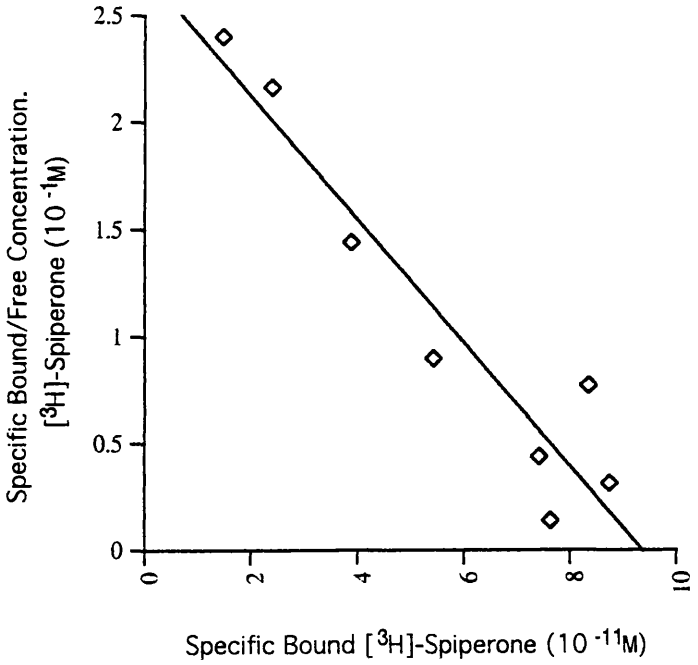
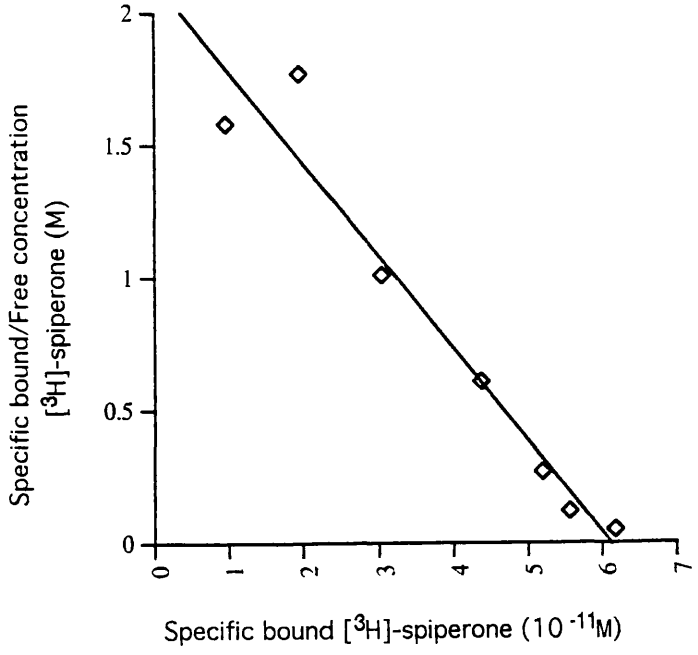


Figure 6.413 Scatchard plot of rat striatal D_2 dopamine receptors binding [^{3}H]-spiperone



binding model in agreement with previous studies (Woodward *et al* 1996; Guiramand *et al* 1995; Leysen *et al* 1992).

Non-specific binding to both receptor preparations was defined using (+)-butaclamol (10^{-6} M) and represented less than 30% of total binding (at Kd concentration of [^{3}H]-spiperone). Saturation assays on wild-type rat-1 fibroblast cells showed no specific binding of the radioligand.

From this specific saturation binding data, the B_{max} (quantity of receptors present) and the dissociation constant (K_D) of the radioligand were calculated for each homogenate (Table 6.416).

Table 6.416 *K_D and B_{max} values for striatal and cloned D2 receptors binding [^{3}H]-spiperone*

	Rat Striatal D_2 Receptors		Mouse D_{2S} Receptor	
Assay Number n	**K_D** (pM)	**B_{max}** (fmol/mg protein)	**K_D** (pM)	**B_{max}** (Fmol/mg protein)
1	21.9	176	28.0	36
2	32.0	255	25.4	25
3	38.0	218	29.6	29
Mean ± S.E.M.	**30.6** ± 4.7	**216** ± 23	**27.7** ± 1.2	**30** ± 3

The dissociation constants (K_D) of both receptor preparations for [^{3}H]-spiperone were very similar (ie not statistically different) in each case (Table 6.416), and agreed with those determined by Falardeau et al (1994), (human D_{2L} = 34 ± 13 pM; D_{2S} = 30 ± 12 pM) and Woodward *et al* (1996), (rat D_{2L} = 33 ± 8 pM). K_D values determined by other groups range from 20-90 pM higher, which could be due to slight differences in assay conditions, however they were still generally in agreement with these values (Castro & Strange 1993; Leysen *et al* 1992; Montmayeur *et al* 1993).

The B_{max} value for the D_{2S} receptors (30 ± 3) is 7 fold lower than for the striatal D_2 receptors (216 ± 23) (Table 6.416). Both values are lower than expected and significantly lower than B_{max} values obtained by other groups.

6.42 Receptor Number

By using the cell count (number of transfected cells used), the specific dpm at saturation, the specific activity of the radioligand and 'Avagadros' Number', the number of cell surface expressed D_2 receptors can be calculated:

Number of cells used in saturation assay = 1.133×10^8 (ie 1/3 of total cells, p.8)

Specific dpm at saturation (approx.) = 1000 Spec. Activity [^{3}H] = 95 Ci/mmol

Avagadro's number = 6.023×10^{23}

$$(2.197 \times 10^{12}) \times 95 = 2.09 \times 10^{14} \text{ dpm/mmol} \quad \text{(equation 1)}$$

$$2.09 \times 10^{14} = 208.7 \text{ dpm/fmol}$$

$$\frac{\text{specific dpm}}{\text{dpm/fmol}} = \frac{1000}{208.7} = 4.79 \text{ fmol receptor/1.9} \times 10^6 \text{ cells}$$

$$\frac{(4.79 \times 10^{-15})}{1.9 \times 10^6} \times (6.023 \times 10^{23}) = \underline{\underline{1500 \text{ receptors/cell}}}$$

6.43 Competition of [^{3}H]-spiperone binding

A series of dopamine antagonists (spiperone, (+)-butaclamol & (-)-butaclamol) and agonists (dopamine, quinperole & 7-OH DPAT) were tested for their ability to inhibit radioligand binding to D_2 receptors in rat striatum and also cloned D_{2S} receptors in rat-1 fibroblast cells. The agonists were tested both with and without the addition of Gpp(NH)p (a non-hydrolysable GTP analog). Tables 6.431 & 6.432 (p74) show the individual drug pK_i values (inhibition constant) and mean slope values (n_H) for the D_2 receptors in rat striatum and the cloned D_{2S} receptor, respectively.

Table 6.431 Binding of agonist and antagonist competitors at rat striatal D_2 receptors

	pK_i				nH
Compound	n=1	n=2	n=3	X ± SEM	X ± SEM
(+)butaclamol	9.02	9.06	9.38	9.15 ± 0.11	0.96 ± 0.02
(-)butaclamol	5.58	5.57	5.58	5.58 ± .003	1.14 ± 0.03
spiperone	9.86	10.08	9.57	9.84 ± 0.15	0.84 ± 0.13
quinpirole - Gpp	6.20	6.43	6.17	6.27 ± 0.08	0.69 ± 0.02
quinpirole + Gpp	5.67	5.90	5.54	5.70 ± 0.10	0.63 ± 0.03
7 OH DPAT - Gpp	6.73	6.79	6.48	6.67 ± 0.09	0.84 ± 0.06
7 OH DPAT + Gpp	6.20	6.28	6.31	6.26 ± 0.03	0.87 ± 0.06
dopamine - Gpp (n = 6)	6.42 6.56	6.25 6.66	6.11 6.78	6.46 ± 0.23	0.70 ± 0.12
dopamine + Gpp	6.01	5.76	5.92	5.90 ± 0.07	0.81 ± 0.08

Table 6.432 Binding of agonist and antagonist competitor compounds at mouse D_{2S} receptors

	pK_i				nH
n	1	2	3	X ± SEM	X ± SEM
(+)-butaclamol	9.50	9.23	9.24	9.32 ± 0.09	0.81 ± 0.02
(-)-butaclamol	5.70	5.76	5.56	5.67 ± 0.07	1.15 ± 0.16
spiperone	10.06	9.90	9.38	9.78 ± 0.21	0.93 ± 0.14
quinpirole - Gpp	5.74	5.66	5.83	5.74 ± 0.05	0.86 ± 0.06
quinpirole +Gpp	5.13	5.13	5.34	5.20 ± 0.07	0.99 ± 0.02
7 OH DPAT - Gpp	6.61	6.26	6.56	6.48 ± 0.11	0.77 ± 0.02
7 OH DPAT +Gpp	6.07	6.07	6.16	6.10 ± 0.03	0.99 ± 0.04
dopamine - Gpp (n = 6)	5.88 6.03	5.43 6.70	6.05 6.17	6.04 ± 0.38	0.68 ± 0.09
dopamine +Gpp	5.18	5.15	5.31	5.21 ± 0.05	0.89 ± 0.03

[+ Gpp = Gpp(NH)p present in assay; - Gpp = no Gpp(NH)p present]

The antagonists all produced very similar results for both mouse D_{2S} and rat striatal D_2 receptors. Spiperone, (+) and (-)-butaclamol gave almost identical pK_i values for each receptor preparation (≈ 9.8, ≈ 9.2 & ≈5.6 respectively).

All agonist compounds had a lower affinity (pK_i) for the cloned mouse D_{2S} receptor than for the rat striatal D_2 receptors, and the addition of Gpp(NH)p also reduced the pK_i value for all of the agonists. The greatest difference in pK_i was

for the endogenous agonist dopamine in the presence of Gpp(NH)p. With the rat striatal D_2 receptor, dopamine has a pK_i 12% higher than with the cloned D_{2S} receptor. Quinpirole also produced a pK_i value 9% higher, for the rat striatal D_2 receptor.

As mentioned, the addition of the GTP analogue Gpp(NH)p to the competing agonist assays reduced pK_i values in all cases (seen as a shift to the right in the binding curve). The mean pK_i values for quinpirole in both striatal D_2 receptor and cloned D_{2S} receptor assays were reduced by 9% each and for 7-OH DPAT by 6%. The largest different in pK_i was for dopamine, where the addition of Gpp(NH)p produced a 10% reduction in its affinity for the striatal D_2 receptors and a 16% reduction for D_{2S} receptors.

From the competition curves produced (Figures 6.433 & 6.434, p77a & b) it can be seen how the shape of the curve for agonists and antagonists varied. The curves were sigmoidal, with the agonists generally producing a shallower curve (slightly lower nH values). The nH values (slope factors) were generally close to 1.0 (unity), consistent with one site binding, however, lower values were obtained for quinpirole with striatal D_2 receptors (0.63), and for dopamine with both receptor preparations (0.68 - 0.7), (Tables 6.431 & 6.432, p74).

6.44 Statistical analysis of competition assay results

The pK_i values (negative log K_i) for each competitor compound with each receptor preparation was used to compare binding at the mouse D_{2S} receptor with that at the rat striatal D_2 receptor in order to determine whether there is a significant difference in pharmacological profile between the two.

The non-parametric Mann-Whitney-Wilcoxon rank sum test was used to test the null hypothesis that 'the median pK_i values of the two D_2 receptor preparations are equal', for each competitor compound.

Table 6.441 Mann-Whitney-Wilcoxon analysis of pKi values between striatal D_2 and cloned D_{2S} receptors

Compound	*P* level
(+)-butaclamol	0.30
(-)-butaclamol	0.50
spiperone	0.50
- GTP quinpirole	0.05
+ GTP quinpirole	0.05
- GTP 7 OH DPAT	0.30
+ GTP 7 OH DPAT	0.05
- GTP dopamine	0.50
+ GTP dopamine	0.05

Significance was determined at the 5% probability level ($P \leq 0.05$).

From Table 6.441 it can be seen that for the antagonists, pK_i values for rat striatal D_2 and cloned mouse D_{2S} receptors are not significantly different therefore the null hypothesis is accepted.

The binding of the agonists however, all show significant results. In the presence of Gpp(NH)p, all three agonists (quinpirole, 7-OH DPAT, and dopamine) showed significantly different pK_i values for the two D_2 receptor preparations (Table 6.441). Though, in the absence of Gpp(NH)p the situation was slightly different, as dopamine and 7-OH DPAT did not produce significant values.

Table 6.442 Mann-Whitney-Wilcoxon analysis of pKi values for D_{2S} receptor in the presence and absence of GTP

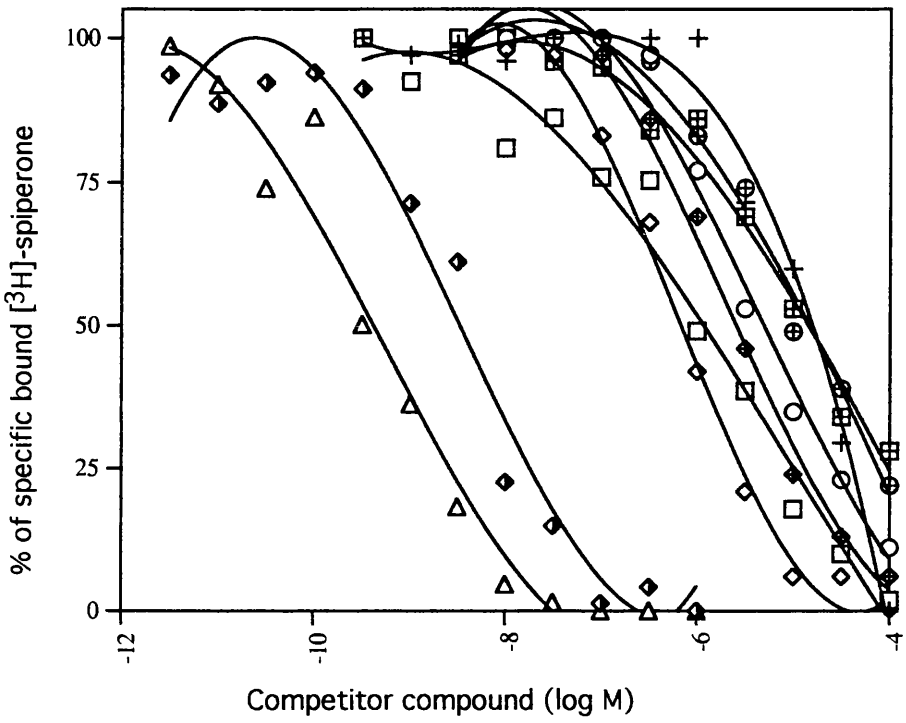
Compound	*P* level
quinpirole	0.05
7 OH DPAT	0.05
dopamine	0.04

Table 6.443 Mann-Whitney-Wilcoxon analysis of pKi values for striatal D_2 receptor in the presence and absence of GTP

Compound	*P* level
quinpirole	0.05
7 OH DPAT	0.05
dopamine	0.02

Comparison of pK_i values was also made for each agonist in the presence and absence of Gpp(NH)p at the same receptor (Tables 6.442 & 6.443). This analysis showed a significant difference for all agonists with both receptor preparations. The endogenous agonist dopamine produced the greatest significance for binding in the presence verses binding in the absence of Gpp(NH)p, with pK_i values giving $P \leq 0.04$ for D_{2S} and $P \leq 0.02$ for striatal D_2 receptors.

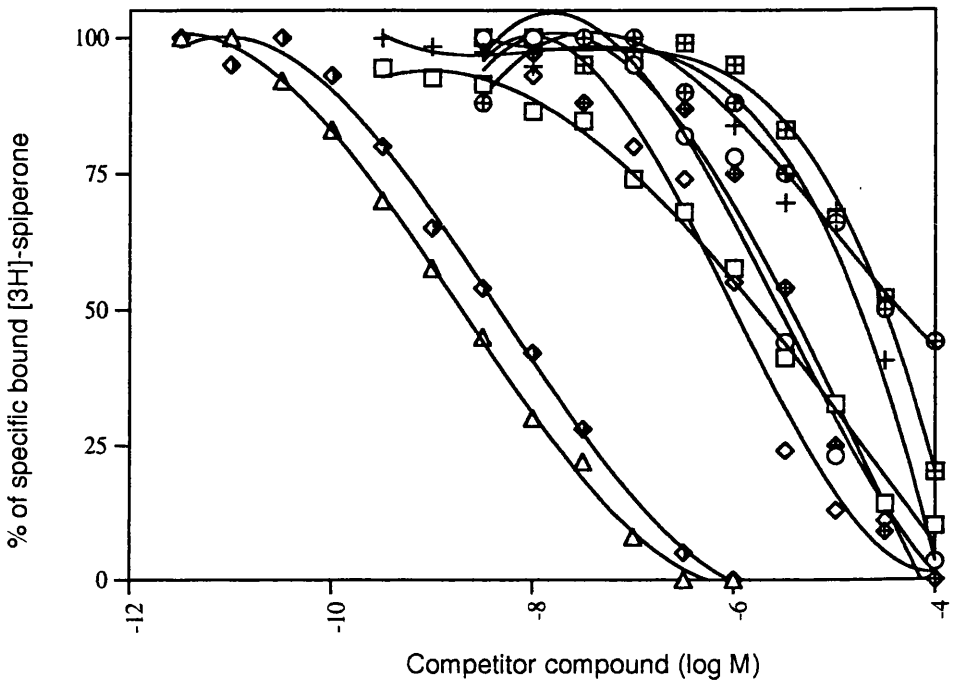
Figure 6.433 Competition curves of all compounds for the binding of [^{3}H]-spiperone at striatal D_2 receptors



Key:-

◆	(+)-butaclamol		
+	(-)-butaclamol		
△	spiperone		
□	dopamine	⊞	dopamine +GTP
◇	7-OH DPAT	◈	7-OH DPAT +GTP
○	quinpirole	⊕	quinpirole +GTP

Figure 6.434 Competition curves of all compounds for the binding of [^{3}H]-spiperone at cloned D_{2S} receptors



+	(-)-butaclamol
□	dopamine
△	spiperone
○	quinperole
⊕	quinperole +Gpp(NH)p
◆	7-OH DPAT +Gpp(NH)p
◇	7-OH DPAT
⊞	dopamine +Gpp(NH)p
◆	(+)-butaclamol

6.5 Discussion of pharmacological results

The aim of these experiments was to examine the pharmacology of a cloned short isoform of the D_2 dopamine receptor (D_{2S}) isolated from mouse striatal tissue, and compare this to the pharmacology of the native population of D_2 receptors in rat striatal tissue.

In order to do this the D_{2S} receptor was stably expressed in rat-1 fibroblast cells. This cell line was selected because it was readily available and had been regularly, successfully used for cellular expression of cell surface receptors by Syntex Ltd. Saturation assays using the radiolabel [^{3}H]-spiperone on membrane preparations of the wild-type cells showed no specific binding.

Radioligand binding was performed on membrane preparations of the transfected cultured cell line and also, in parallel, on membrane preparations of striatal tissue from Sprague-Dawley rats. Allowing analysis of the pharmacological profile of the D_2 receptor population normally expressed in this region of the rat brain.

This area of the brain was selected as it has been shown to contain the highest abundance of D_2 receptors and would be expected to consist of approximately 25% short isoform to 75% long isoform (Giros *et al* 1989).

Rat and mouse have very similar D_2 receptor sequences with very few differences at the amino acid level (figure 5.11, p52a) and similar binding profiles making them useful for comparison.

Spiperone is an antagonist of D_2 receptors commonly used in binding experiments. The tritiated nature of the drug does not affect its binding but provides a reasonable specific activity and a long half-life. Its specificity is

shown by its displacability by other D_2 receptor specific drugs, as illustrated in the competition assay. [^{125}I]-iodospiperone is also sometimes used as it has a higher specific activity but a much shorter half-life.

6.51 Saturation binding

In figures 6.412 & 6.414 (p71a) it can be seen that the specific binding of [^{3}H]-spiperone of D_2 receptors is saturable in both membrane preparations. The Scatchard plots (figures 6.413 & 6.415, p71b) are linear indicating that the binding is at one site, ie one receptor type. In the case of the D_{2S} receptor clone saturation occurs at a ligand concentration which is a factor of 10 lower than for the striatal receptors. The B_{max} values are 10 fold lower, however the K_D values are the same.

The B_{max} is the amount of receptor present in the preparation as femtomoles per microgram of protein. The values of 216 (± 23) for rat striatal tissue and 30 (± 3) for the expressed mouse D_{2S} clone were both low compared with published levels (Leysen *et al*, 1992; Montmayeur *et al* , 1993), possibly due to differing assay conditions.

Expected B_{max} levels were based on results obtained by other groups. It could be expected that receptor density would be consistent in rat striatal tissue, however Leysen *et al* (1992) reported a level four times higher (864 ± 146 fmol/mg). The clone also had a very low B_{max} with a value almost seven fold lower than that demonstrated by Montmayeur *et al* (1993) for a mouse D_{2S} receptor clone, even though the K_D values in each case were comparable.

Montmayeur's group used the eukaryotic expression vector pSG5 to transiently transfect JEG3 (human carcinoma) cells. Whilst in this experiment pcDNA3 was stably transfected into rat-1 fibroblasts.
Could the problem be caused by the type of transfectants analysed or the use of a cell line from a different species? This doesn't appear to be the case, as similar research performed by other groups shows the range of different expression methods used.

Leysen *et al* (1992) used stable transfectants of human D_2 clones in human embryonic kidney cells and produced good B_{max} levels. Meanwhile Castro & Strange (1993) obtained a high B_{max} by expressing the rat D_{2L} clone in mouse fibroblast cells. Therefore the result here appears not to be due to expression in a different species cell line or the difference between stably or transiently transfected lines. However, it has been shown that the same D_2 receptor clone expressed in two different cell lines gives different binding profiles (Castro & Strange, 1993[2]), therefore it is difficult to compare the results of any two groups using different expression systems.

A possible reason for the low expression is that the clone may require upstream sequences for a high level expression. The rat D_{2L} clone used by Castro & Strange (1993) is a 2.5 kb cDNA containing the gene which is actually only $\approx$ 1.3 kb, whereas the cDNA expressed in this experiment is solely the ORF (open reading frame) of the gene.

Also the expression was stable therefore the vector construct had integrated into the cell genome (facilitated by the SV40 sequences of the vector). It is possible therefore that the insertion event had occurred at a region of suppressed expression such as a region of heterochromatin. However $\geq$40 transfected lines were tested and all gave relatively low radioligand binding, the line used in the assays had the

highest B_{max} therefore it is unlikely that all lines had suppressed expression unless there was a problem with the vector, which was not apparent.

The K_D value for the cloned D_{2S} and striatal D_2 receptors were very similar and agreed with those previously determined by Falardeau *et al* (1994), (human D_{2L} = 34 ± 13 pM; D_{2S} = 30 ± 12 pM) and Woodward *et al* (1996), (rat D_{2L} = 33 ± 8 pM). However K_D values determined by other groups range from 20-90 pM higher, (Castro & Strange 1993; Leysen *et al* 1992; Montmayeur *et al* 1993) for various D_2 and D_{2S} clones. The variance is probably due to slight differences in assay conditions, but other groups also noted the similarity of K_D between the two D_2 receptor isoforms (Falardeau et al, 1994; Castro & Strange, 1993)

6.52 Competition Binding

Several different agonists and antagonists were investigated for their ability to inhibit the specific binding of [^{3}H]-spiperone to both the striatal and cloned D_2 receptor preparations.

For each compound at each receptor the IC_{50} was calculated, (concentration of competitor compound which causes inhibition of 50% specific radioligand binding). This was then converted to K_i (equilibrium dissociation constant) using the Cheng-Prusoff equation, allowing comparison of the pharmacology of competitors as it is independent of ligand concentration; and subsequently to pK_i (negative log K_i) which provides a whole number index of relative affinity of a compound for a receptor sample allowing easy reference and statistical comparison.

The nH value (slope factor) indicates the slope of the curve which were generally close to 1.0 (unity), consistent with one site binding. Values less than unity indicate, in this case, interconverting receptor states.

6.53 Statistical Analysis

The antagonist compounds spiperone, (-)-butaclamol and (+)-butaclamol gave pK_i values that were not significantly different for each of the receptor preparations, which agrees with results from other groups (Table 6.531).

Table 6.531 Comparison of pK_i values for D_2 antagonist compounds

Compound	pK_i (nM)		Reference
	D_{2Long}	D_{2Short}	
Spiperone	10.2	10.1	Montmayeur et al., 1993
	10.2	10.4	Castro & Strange, 1993
	10.8	11.0	Leysen et al., 1993
(+)-Butaclamol	9.0	8.9	Grandy et al., 1989
	8.4	8.4	Castro & Strange, 1993
	10.2	10.3	Leysen et al., 1993

(Source: Falardeau 1994)

These and other groups who have investigated antagonist binding have also not discovered any significant differences in binding of antagonists between the D_2 receptor isoforms, suggesting that the two forms have very similar, if not identical antagonist binding sites.

The agonists however, produced some interesting results. Agonist compounds as discussed in the introduction have their binding affinities affected by the 'state' of the receptor. The normal situation in a membrane preparation is that the receptors are a population of different affinity states; some are coupled to G-proteins and therefore in the 'high-affinity' state whereas others are dissociated and at 'low-affinity'. The population is dynamic with constant cycling of G proteins and GTP

hydrolysation. This complicates the analysis of binding results as ratios of states can differ between assays.

Therefore the addition in this experiment of excess non-hydrolysable GTP analogue Gpp(NH)p disrupts this equilibrium and converts all binding sites to the low-affinity state.

Addition of Gpp(NH)p reduced the pIC_{50} for each compound. This occurred the low affinity binding state requires a higher concentration of ligand to inhibit the radioligand binding (increased IC_{50}). This can be see by the shift to the right of the agonists competition curves for the Gpp(NH)p assays (Figure 6.433 & 6.434, p77a & b). The fact that this occurs in the case of the clone, shows that the D_{2S} receptor appears to be coupling with endogenous cellular G-proteins.

This decrease in pIC_{50} was significant for all three agonists both for the cloned D_{2S} and striatal D_2 population (Tables 6.442 & 6.443, p77a & b) which indicates that the receptor state does influence affinity for agonists (Birnbaumer *et al*, 1990)

The results of the Gpp(NH)p assay were then statistically analysed to compare the binding of each agonist between each receptor preparation. The results show a difference in agonist affinity for the striatal D_2 and D_{2S} receptor groups.

All of the agonists tested had significantly lower pIC_{50} values for the mouse D_{2S} receptor clone. The lower binding affinity of the agonists for the cloned receptor when compared to striatal D_2 receptors agrees with the findings of Leysen *et al.* Their study revealed that dopamine and quinpirole had lower affinity for expressed clones of both the short and long isoform of the human D_2 receptor when compared to rat brain tissue preparations. They discovered that "agonists showed an apparent lower affinity for the cloned receptor than for the striatal form" with

IC_{50} values differing by up to 6 fold. pIC_{50} values of dopamine for the cloned receptor were 4-10x lower than for striatal tissue.

Their justification for this was that there may be a difference in the proportion of coupled:uncoupled receptors in each case, ie there was less coupling in the case of the cloned receptor due to coupling being less efficient or the appropriate G proteins weren't present in the chosen cell-line. However this does not explain the difference for binding in the presence of Gpp(NH)p as the point of this assay is to remove the influence of G protein coupling upon receptor affinity state.

It could simply be that the receptors perform better in their native situation; that uncoupling is not totally effective in tissue preparations because of extra material impeding the compounds diffusion; or that there is a genuine difference in agonist binding between the preparations.

Another possibility is that the difference in B_{max} between the striatal receptors and the cloned D_{2S} receptors could have affected the result. Castro & Strange (1993[2]) suggest that differences in expression levels of receptors could affect results because of receptor/G-protein ratio in the cell. Also in this case different G-protein populations are likely to have been present in the membrane preparations due to the different cell types involved. However, these differences involving G-proteins should have been eliminated by the addition of Gpp(NH)p and receptor/G-protein dissociation.

In another study on the pharmacology of dopamine receptors, no significant difference in binding was found between the cloned D_{2S} & D_{2L} isoforms (Falardeau et al, 1994). It seems to be that a difference in ligand interaction would not be expected, as the difference (the 29 residue spliced region in the third intracellular loop) is on the cytoplasmic side of the membrane and quite discrete

from the sites implicated in ligand binding (figure 1.3, p6a). However, Castro & Strange (1993) discovered that substituted benzamide antagonists had significantly higher affinity for D_{2S} dopamine receptors and proposed that difference was due to an overall conformational change caused by the 29 amino acid insertion in the D_{2L} dopamine receptors interacting with the transmembrane regions in some way.

A difference between the two isoforms which has been discovered involves the spliced region. This segment appears to be involved in the D_{2L} selective recognition of a specific inhibitory G protein α-subunit (Gαi2) and suggests the possibility of the two isoforms performing separate functions by activating different G-proteins and transduction pathways (Guiramand *et al* 1995).

6.6 Conclusion

The mouse D_{2S} dopamine receptor has been successfully cloned and expressed and behaves much as expected according to previous research.

Both the D_{2S} receptor clone and the striatal D_2 receptors showed coupling to G-proteins and displayed different affinity states for agonist compounds. Addition of a non-hydrolysable analogue of GTP shifted both receptor preparations to a uniform low-affinity state.

In comparison with striatal D_2 receptors the D_{2S} clone binds antagonists with the same affinity but appears to have greater affinity for agonist compounds. A similar result was shown by Leysen et al, (1992) who also used rat striatal D_2 receptors as a comparison and discovered that cloned D_2 receptors had a lower affinity for agonists. Unfortunately both isoforms of the D_2 receptor displayed lower affinity than striatal D_2 receptors, but the difference in affinity for agonists between the clones was negligible.

This suggests that striatal material was not the best comparison to look at differences between the long and short isoform and ideally a D_{2L} receptor clone should have been used (which was not available). However it does show a difference in binding profiles between a cloned receptor and those in their native environment suggesting that in isolation a receptor may behave differently from its native situation. This factor is important when considering the effects of drugs in pharmacological research.

The methods, equipment and materials (with the exception of the clone) were provided by and routinely used in the research laboratories of Syntex Ltd., Heriot-Watt Research Park, and therefore were established and validated.

SECTION B

CHAPTER 7

DISCUSSION

7.0 Discussion

Although not all of the aims which were set out at the start of this project have been reached, much has been achieved in the time available. The ground-work laid during the work for Section A, proved very useful in designing and carrying out the research in Section B.

The dopamine receptor area has been reasonably well researched and therefore I had a solid foundation on which to base my research and to compair my research. Although the stage of creating chimeras was not reached, this is still a technique of great potential in investigating binding sites. Even though the D_3 receptor was not cloned it would have still been possible to incorporate regions of D_3 sequence within recombinant primers or by point mutations, to determine key residues. Unfortunately, this stage was not reached within the time, however, it is still a valid project.

The mouse dopamine D_{2S} receptor was successfully cloned and expressed, allowing investigation of its pharmacology and comparisons with previous studies. Although no significant differences were found, it was noted that there is a significant difference in binding profiles between a receptor when cloned and when in its native environment, even though experiments were carried out in standard physiological conditions.

The fact that in isolation a receptor may behave differently from its native situation is important when considering the effects of drugs in pharmacological research, as the ligand-binding assays used in this study tend to be standard for industrial research.

Future Research

If there had been time, much more work could have been carried out on the mouse D_{2S} dopamine receptor clone. At the time that I finished, work had already begun on designing and making mutants of the D_{2S} receptor, which would have been pharmacologically profiled in the same way and the results compared to the wild type receptor.

Ultimately it was anticipated that the mouse D_3 dopamine receptor would also be cloned and this receptor could also be profiled. My particular interest would be to mutate key regions shown to be involved in ligand binding on the D_2 dopamine receptor into the corresponding residues on the D_3 dopamine receptor and vice versa, in order to locate the exact regions/residues which differentiate each receptor's individual pharmacological properties.

It would also be interesting to repeat this experiment with a D_{2L} receptor clone as a comparison. Further ligands could be tested on the D_{2S} dopamine receptor, particularly substituted benzamide antagonists (eg raclopride and remoxipride) which have been reported to have a higher affinity for the D_{2S} receptor isoform over the D_{2L} receptor.

CHAPTER 8

METHODS

8.1 Introduction of plasmid DNA into *E. coli*

8.11 Preparation of competent cells (Hanahan, 1983)

An overnight culture of the recipient strain (grown in 2YT broth) was diluted 1 in 100 into 10ml 2YT broth and incubated at 37°C for 90-120 minutes to a density of approximately 10^8/ml (OD_{600} 0.45-0.55). The cells were harvested by centrifugation (12000g, 10 mins, 4), resuspended in 1ml ice cold TFB (10mM MES/KOH pH 6.3; 100mM RbCl; 45mM $MnCl_2$; 10mM $CoCl_2$; 3mM hexaminecobaltic chloride) and incubated on ice for 15 mins. Next 34µl DMF was added and the cells incubated on ice for 5 more minutes, then 34µl β-mercaptoethanol (14.4M) and after a further 10 mins on ice another 34µl DMF. The cells remained on ice until use (the same day).

8.12 Transformation procedure

Transformations were carried out in sterile 1.5ml microfuge tubes. 1-5µl of ligation mix was added to 100-200µl of competent cells and incubated on ice for 45 mins. The tubes were then heat-shocked at 42°C for 3 mins and returned onto ice. The cells were then transferred into sterile universal tubes containing 1ml 2xYT broth and incubated at 37°C for 30 mins. After this time, 200µl of each cell suspension was plated on LB plates containing appropriate antibiotic and chromogenic substances and incubated at 37°C overnight.

8.13 Selection of pUC-derived recombinant clones

8.131 Antibiotic

A stock solution of ampicillin (20mg/ml in water) was added to molten agar (@ 55°C) to a final concentration of 50µg/ml.

8.132 Chromogenesis

Used in conjunction with IPTG this was used to identify *E. coli* strains containing pUC based vectors with inserts in their multiple cloning sites. Colonies of recombinants are generally white, whilst those lacking inserts are blue. From a stock concentration of 20mg/ml in DMF, X-gal is used at a final concentration of 20μg/ml and IPTG from 24mg/ml in dH_2O stock is used at 50μg/ml on L-agar plates.

8.14 Glycerol storage of bacterial cultures

850ml of a fresh overnight culture of the strain (grown with antibiotic if necessary) was placed in a 'Nunc' tube (Gibco BRL) with 150ml sterile glycerol and vortexed. The tube was then labelled and quick frozen in liquid nitrogen before storage at -70°C. When required, the surface of the frozen culture could be scraped with a sterile loop, streaked onto appropriately prepared agar plates and incubated inverted at 37°C.

8.2 DNA Purification

8.21 Preparation of plasmid DNA

A 1.5ml aliquot of each overnight culture of the *E. coli* strain containing the transformed plasmid, was taken in a sterile 1.5ml microfuge tube. The cells were then pelleted by centrifugation at high speed in a microcentrifuge. The supernatant was removed and the cells resuspended in 200μl of cell resuspension buffer (50mM Tris-HCl, pH 7.5; 10mM EDTA; 100μg/ml RNAse A). To this 200μl of cell lysis solution (0.2M NaOH; 1% SDS) was added and the tube inverted to mix until the suspension cleared. Then 200μl of neutralisation solution (2.55M KCOOH, pH 4.8) was added and the tube again inverted to mix. The tubes were then microcentrifuged again at high speed for 10 mins and the sticky pellets produced were removed with sterile toothpicks.

To the clear supernatant remaining 750µl of Wizard™ Miniprep Purification Resin (Promega) was added and mixed by inversion. This mixture was then drawn through a Vac-Man™ Laboratory Vacuum Manifold (Promega) and followed by 2ml Column Wash solution (200mM NaCl; 20mM Tris-HCl, pH 7.5; 5mM EDTA; dilute 1:1 with 95% ethanol). The resin was dried by continuing to draw a vacuum through the column for a further 2 mins then the column was transferred to a 1.5ml microcentrifuge tube and spun at high speed for 20 sec to remove all residual Column Wash solution.

The column was then transferred to a fresh sterile microcentrifuge tube and 20µl of 1x TE buffer (@ 70°C) applied to the top of the column. After 1 minute the column and tube were spun at high speed for 30 sec. This process was repeated, and the solution collected in the microfuge tube. The column was discarded and the DNA solution stored at -20°C.

Before use the samples were analysed on a 1% agarose gel, in order to quantify and to check for contamination.

8.3 Dideoxy DNA Sequencing

8.31 Denaturation of double stranded DNA template

DNA was prepared as in method 8.1, then 18µl was taken in a sterile microfuge tube, 2µl NaOH (2M) was added and the tube incubated at 37°C for 10 mins. Next 50ng of the appropriate sequencing primer was added along with 6µl KCOOH (2.55M, pH 4.8) and the tube vortexed to mix. 180µl of 95% ethanol was added and the tube vortexed, then microcentrifuged at high speed for 15 mins. The supernatant was carefully removed and 500µl 70% ethanol added before recentrifuging. The supernatant was again removed and the tube dried by incubation at 37°C.

8.32 Single strand DNA template production

An alternative method of producing DNA template for the sequencing reaction involved using the helper phage M13K07 (Promega).

The required fragment was cloned into a suitable vector containing an origin of single stranded replication (see cloning, method 8.7) and transformed into a F' host strain.

In a sterile universal tube 1.5 ml 2YT broth (bacto-tryptone; bacto-yeast extract; NaCl) containing M13K07 helper phage at 10^7 pfu/ml and ampicillin at 200µg/ml, was inoculated with a single fresh colony of the clone. This was incubated in a shaking incubator at 37°C for 2 hr then kanamycin was added to 75µg/ml and the culture was return to the incubator for a further 18 hr.

After this time the culture was transferred to a microfuge tube and spun at 15,000 rpm. The supernatant was transferred to a new tube, 200µl PEG solution added (33mM polyethylene glycol 6000; 0.5M NaCl) and vortexed before incubating at room temperature for 20 mins. The mixture was then spun at 15,000 rpm for 5 mins and the PEG solution discarded. Care was taken not to dislodge the pellet but to totally remove the supernatant by pulse spinning the tube and removing the residual PEG solution by vacuum suction through a drawn out Pasteur pipette.

The phage pellet was resuspended in 100µl 1xTE (10mM Tris-HCl, pH7.5; 1mM EDTA), an equal volume of buffer saturated phenol added and the tube vortexed. After 5 mins at room temperature it was again vortexed and spun at 15,000 rpm for 2 mins. The upper aqueous layer was transferred to a new eppendorf tube, 2.5 volumes ethanol and 0.1 volumes NaCOOH (3M) were added and the DNA precipitated at -70°C for 1 hr.

The tube was spun at full speed again for 15 mins and the pellet washed in 95% ethanol. The ethanol was removed and the pellet dried before resuspending in 30µl 1x TE, the single stranded DNA could then be used or stored at -20°C.

8.33 Sequencing Reaction

The denatured/single stranded DNA template was resuspended in 7µl dH_2O and 2µl annealing buffer (200mM Tris-HCl, pH7.5; 100mM $MgCl_2$; 250mM NaCl), then 10ng of the appropriate primer was added. This was incubated at 37°C for 10 mins then kept on ice.

As a mixture; 1µl DTT (100mM), 1.5µl dH_2O and 5µCi [α^{35}S]dATP were added to the tube and vortexed before replacing on ice. DNA polymerase (Sequenase 2.0, USB) was diluted 1:8 with ice-cold enzyme dilution buffer (10mM Tris-HCl, pH7.5; 5mM DTT; 0.5mg/ml BSA) to 1.5units/µl.

3 units of DNA polymerase was added to the reaction tube containing the DNA and annealed primer and mixed carefully. The labelling reaction was allowed to proceed at room temperature (15-20°C) for 2-5 mins before termination was begun.

Four 0.5ml microfuge tubes were incubated at 42°C. Each contained 2.5ml of one of the dideoxy termination mixes (80µM dGTP; 80µM dATP; 80µM dCTP; 80µM dTTP plus 8µM dideoxy-dNTP). After the labelling period, 3.5µl of the reaction was added to each of the four termination tubes and incubated at 42°C for 2-5minutes before 4µl stop solution was added to each termination reaction. The sequencing reactions could then be stored at -20°C or immediately run on an acrylamide gel.

8.34 Acrylamide Gel Electrophoresis

8.341 Preparation of the gel

The gel mix was made by dissolving 420g sequencing grade urea in 250ml dH_2O and 5xTBE, 125ml 40% bis acrylamide (19:1 Severn Biotechnology) was then added and dH_2O to 1litre. The mix was filtered through 3mm filter paper (Whatman) and stored at 4°C in the dark.

8.342 Electrophoresis

The gel was cast using 'Biorad' sequencing apparatus (the plates being pre-siliconised with dichlorodimethylsilane), and run with 0.6x TBE buffer in the reservoirs at 100-120W, 1900V and 50-60mA. The sequencing samples were heated to a temperature of 85°C for 2-3mins then placed on ice whilst the gel was pre-run for several minutes to heat the apparatus. Samples were loaded in a set order, using sharkstooth combs (3µl per well) and run for between 2-6hours depending on the distance of run required.

After the required running time the power was disconnected, the gel transferred to 3mm paper and the exposed surface covered with SaranWrap. The gel was dried on a vacuum gel drier then exposed overnight to X-ray film, after removal of the SaranWrap. The sequence could then be read from the developed film.

8.4 Agarose Gel Electrophoresis

A gel was prepared according to the number and size of products to be analysed. Generally the larger the DNA fragments, the lower the % of agarose used. For example, fragments of >1kb would be best separated on a 0.7% gel, whereas for fragments of <0.6kb a 1.5% gel would be appropriate. Usually a 1% gel was used. This was made by dissolving 1g agarose powder in 99ml 1xTAE buffer by warming in a microwave, then 50µg ethidium bromide was added before pouring

into the gel former. When set the gel was run in 1xTAE buffer. The length of time required for the DNA to migrate depended upon the voltage across the gel and its length.

After electrophoresis the DNA was visualised using a UV transilluminator. This allowed photographs to be taken for further reference.

8.5 DNA isolation

8.51 Activation of NA45 paper (Schleider & Schuell)

The paper was handled carefully using forceps and cut into strips of 5mm width. These were then washed in 10mM EDTA for 10 mins and then transferred to 0.5M NaOH for a further 5 mins. Finally the paper was rinsed rapidly in dH_2O and stored at 4°C submerged in dH_2O.

8.52 DNA Isolation

DNA was firstly run on an agarose gel and visualised under low wavelength UV (to minimise damage to DNA). A cut was made just below the band required using a sterile scalpel blade, and a piece of NA45 paper of the appropriate length inserted. The gel was reintroduced to the electrophoresis apparatus for 2 mins at a voltage of 100-120 then revisualised to confirm DNA transfer had occurred.

The gel was disposed of and the NA45 paper carrying the DNA was washed briefly 3 times in 500µl aliquots of low salt NET, then transferred to a microfuge tube containing 200µl high salt NET and incubated at 68°C for 15 mins. This was repeated for 30 mins in a fresh 100µl aliquot of high salt NET, then the 2 aliquots of high salt NET were combined, along with 100µl dH_2O, 100µl ammonium acetate and 1ml 95% ethanol. The DNA was precipitated at -20°C and resuspended in 5-10µl 0.1% TE. The paper was checked under UV light to confirm that all of the DNA had been eluted.

8.6 Polymerase Chain Reaction (PCR)

8.61 Oligonucliotide deprotection

The synthesised oligonuclitides required deprotection to detach them from the synthesis beads and prepare for use.

Firstly the synthesis column containing the oligo was carefully opened and the beads with the oligos attached collected in a 'Nunc' tube (Gibco BRL). 1ml of fresh 30% ammonium hydroxide was added and the tube incubated at RT for 1-2 hours. After vortexing the tubes were quickly centrifuged and the supernatant transferred to a fresh Nunc tube. A further 1ml fresh 30% ammonium hydroxide was added to this new tube and then incubated overnight at 50°C.

The oligo was precipitated by adding 10% ammonium acetate (w/v) and 2 volumes of 95% ethanol and storing overnight at -20°C before resuspending in 1%TE to 10% of the original volume.

8.62 Hot-Start Thermal Cycling

Single stranded cDNA template produced from mouse brain tissue by the reverse transcription method mentioned previously was used along with the appropriate primer pair for the reaction. In a thin-walled PCR tube (Greiner) the following were mixed; 10ng cDNA, 200pM of each primer, 2mM each dNTP, 10x Taq buffer (Promega), (1.5mM MgCl2; 10mM Tris-HCl, pH9 @ 25°C; 50mM KCl; 0.1% Triton X-100) and the volume made up to 30µl with dH_2O. This mix was heated to 96°C for 5 mins in a Perkin-Elmer 9600 PCR machine. Meanwhile 2 units Taq enzyme (Promega), 10x Taq buffer and dH_2O to increase the volume to 20µl, were mixed and added to the PCR reaction at the end of the 5 minute hot-start, increasing the final volume to 50µl.

The PCR was performed in condensation free tubes however if these tube are not available a wax pellet or drop of liquid paraffin should be used to seal the reaction.

The reaction then proceeded through 25-35 cycles of amplification according to the program used (see appendix II). The basic program involves approximately 30 sec of denaturation ≥ 94°C, up to 1 min at the annealing temperature (approximated at 5°C below the T_m of the primers), and 1-2 mins at 72°C for optimum nucleotide incorporation by Taq. For the specific PCR cycle program used in each case see that chapter or appendix II.

8.7 DNA Cloning

8.71 Ligation

A suitable vector was selected according to various factors including the function of the clone (ie sequencing, expression); available restriction sites; size and nature of insert etc. The pcDNA3 vector was used as an expression vector for the mouse D_{2S} receptor clone. It was firstly cut with the restriction enzyme *Eco RI* which cuts the vector once in the polylinker (5µg vector; 10-20u *Eco RI*; 11µl dH_2O; 2µl 'React 3' buffer (Gibco BRL) at 37°C for 90 mins, at this point 0.5µl Shrimp Alkaline Phosphatase (SAP), was added to the reaction and the tube was kept at 37°C for a further 30 mins. The SAP was then deactivated by heating to 68°C for 15 mins.

8.711 'Ready-To-Go' Ligation Kit (Promega)

50-250ng vector (5.4kb) was mixed with the 250ng of mouse D2 receptor (1.3kb), (1:4 ratio) in a Promega 'Ready-To-Go' ligation tube. The final volume was made up to 20µl using dH_2O. This was gently mixed and incubated at 16°C for 1 hour.

8.72 PCR products

The pT7Blue plasmid (AMS Biotechnology) is constructed with single 3' dT overhangs, specifically for the purpose of ligation to DNA amplified by DNA polymerase which leaves single 3' dA overhangs.

In a standard reaction 50ng pT7Blue T-vector was ligated with 0.2pmol amplified product in a volume of 10µl. Typically this consisted of combining, in a 1.5ml microfuge tube 1µl 10x ligation buffer (200mM Tris-HCl, pH7; 50mM $MgCl_2$), 0.5µl 100mM DTT, 0.5µl 10mM ATP, 1µl amplified product (~0.2pmol) in 0.1% TE, 4.5µl dH_2O and finally 0.5µl (2-3 units) T4 DNA ligase. This was gently mixed and incubated at 16°C overnight.

A positive control was included by repeating the ligation using 5ng of a 50bp control insert with single 3'dA residues (AMS Biotechnology) which gave an insert to vector molar ratio of 5:1.

8.721 Transformation of pT7Blue(R) constructs

Once ligated, the vector was transformed into an appropriate strain which had been rendered competent.

The standard transformation method (8.2) using TG1cells was generally used, however if a particularly high frequency was required (eg >$4x10^8$) then NovaBlue competent cells (AMS Biotechnology) were used.

The cells were stored at -70°C until required, as 20µl aliquots in 1.5ml microfuge tubes. The cells were then thawed on ice and mixed gently to ensure even suspension of the cells. 1µl of the overnight ligation mix was added to a 20µl aliquot and the tube gently flicked to mix, this was repeated for 1µl of the positive control ligation and 1µl (0.2ng) test plasmid. The tubes remained on ice for a

further 30 mins and were then heat shocked at 42°C for 40 sec and replaced on ice for 2 mins. 80µl of 2YT medium at RT was added and the tubes transferred to incubate at 37°C, shaking at 200-250rpm for 1 hr.

After this time 50µl of each transformation was plated on LB agar plates containing 50µg/ml ampicillin, 15µg/ml tetracycline (to select for F') and IPTG/X-gal for chromogenic selection. The plates were inverted and incubated at 37°C overnight.

8.8 cDNA Synthesis

8.81 RNA extraction from brain tissue

Brain tissue had previously been dissected from freshly sacrificed mice, dissected and frozen to -70°C within mins of the animal's death. Approximately 1g of frozen brain tissue was immersed in liquid nitrogen in a DEPC treated mortar and a pestle used to crush the tissue to a fine powder. More liquid nitrogen was added as required to prevent the tissue thawing. When a powder had been achieved the powder was emptied into a RNAse free 50ml Falcon tube (Corning) and 2ml guanidinium thiocyanate solution (50µl 1M sodium citrate, 0.95g guanidinium thiocyanate, 0.02g Sarcosyl (Sigma)), the tube was inverted to mix. To this mixture 14µl b-mercaptoethanol, 2ml of 2M NaCOOH were added and mixed.

8.82 RNA Extraction

An equal volume (2ml) phenol (H_2O saturated) was added to the tissue mixture and the tube vortexed and incubated on ice, for 20 mins. After this time it was spun at 5k for 10 mins and the upper, aqueous layer removed into a fresh RNAse free universal tube where an equal volume of chloroform/isoamyl alcohol (50:1) was added vortexed and the mixture again incubated on ice for 5 mins. The 5k spin was repeated and the upper aqueous layer again removed into a fresh RNAse

free tube, where two volumes of isopropanol were added and the sample stored at -70°C until required.

When required the sample was aliquoted into 1.5ml microfuge tubes and spun at 15k for 30 mins at 4°C, The pellet produced was washed with 70% ethanol and resuspended in RNAse free TE buffer, before immediate use in the synthesis of cDNA.

Any unused RNA could be stored at -70°C in RNAse free ethanol and NaCOOH (10%).

8.82 cDNA Synthesis

Total RNA extracted from mouse brain tissue was used to produce single stranded cDNA by reverse transcription. 1μg RNA in DEPC treated dH_2O was heated to 90°C for 5 mins and cooled quickly on ice in order to denature any secondary structure. To this 1.5ml microfuge tube, 100pM random hexamers (Gibco BRL); 5mM of each dATP, dGTP, dCTP, dTTP; 1x RT buffer (Promega); 20u RNAsin (Promega) and 5u (0.5μl) Avian Myeloblastosis Virus reverse transcriptase (AMV RT), (Promega), were added and the volume increased to 20μl with dH_2O. This mixture was then heated to 23°C for 10 mins, then 42°C for 45 mins and finally 95°C for 10 mins to stop the reaction. The cDNA was stored at -20°C until use.

8.9 Pharmacological Methods

8.91 Membrane Preparation of Rat Striata

Striata were dissected from whole rat brains (Sprague Dawley, Charles River UK Ltd.) and each pair suspended in 60 volumes (w/v) of buffer (50mM Tris HCl; 120mM NaCl; 2mM $CaCl_2$; 1mM Mg_2SO_4; 1mM EDTA) at 4°C on ice. The buffer was adjusted to pH7.4 which corresponds approximately to the

physiological pH. A Polytron P10 tissue disrupter was washed twice with 500ml distilled water and then used to homogenise the tissue at setting 10, for two 10 second periods. The homogenate was then centrifuged at 48 000g (SS34 rotor in a Du Pont RC5B centrifuge) for 15 mins to wash the membrane preparation and remove any endogenous neurotransmitters.

The supernatant was discarded and the pellet was resuspended in the same amount of fresh buffer using the Polytron P10 disrupter at setting 5 for 5 sec. The tissue was then recentrifuged using the same conditions. This process was repeated twice more.

Finally each paired striatal pellet was resuspended in 3ml buffer using the Polytron P10 disrupter. Care was taken to recover any homogenate adhering to the disrupter by washing with a small amount of buffer.

The membrane preparation was then ready to be used in assays or could be frozen in liquid nitrogen before storing at -70°C.

8.92 Cell Culture and Membrane Preparation

Rat-1 fibroblast cells were transfected with the mammalian expression vector pcDNA3 (Invitrogen) containing the mouse D_{2S} receptor cDNA and grown to a cell density of $\approx 3.4 \times 10^8$. The cell count allowed calculation of the cell number per assay so that receptor number per cell could be determined. The cells were then harvested in phosphate buffered saline (1x PBS) containing 2mM EDTA. (This work was performed by Mr Bill Rowand of Syntex Research, Scotland.)

The cells were then homogenised and washed in the same way as the striatal preparations. These were resuspended in 6ml buffer and 2ml ($\sim 1.1 \times 10^8$ cells) was used per assay. This was then diluted 1:15 with buffer ($\sim 7.5 \times 10^6$ cells/ml) and 250µl cells used per assay tube ($\sim 1.9 \times 10^6$ cells/tube).

8.93 Saturation Assay

Triplicate total binding tubes, duplicate NSB tubes and duplicate total counts (unfiltered) tubes were used in order to reduce handling error, the experiment was carried out three times (n=3) and means and standard errors (S.E.M.) were calculated. The radioligand was diluted over a range of concentrations which was calculated to span from approximately 10 fold higher to 10 fold lower than the K_D. The highest concentration of ligand ([^{3}H]-spiperone, 95Ci/mmol, Amersham) was 2nM (1mM stock diluted 1:500, ie 6µl in 3ml assay buffer) plus 7(1:1) serial dilutions to 0.016nM.

Assays were set up in 4.9ml Sterilin assay tubes with constituents added in the order and quantities shown in Table 8.931.

Table 8.931 Saturation Assay Composition

	total binding	NS binding	total counts
Buffer	100	50	100
Buffer + 10^{-7} M ketanserin	100	100	100
[^{3}H]-spiperone	50	50	50
Membrane homogenate	250	250	250
(+)butaclamol (10^{-6}M)	-	50	-
TOTAL VOLUME	500µl	500µl	500µl

The assays were then vortexed and incubated at 37°C (physiological temperature) for 30 mins before being filtered over Whatman GF/B (glass fibre) filters. The total count tubes (unfiltered) were spotted onto the filters in 25µl aliquots (duplicated for each tube). The total binding and NSB tubes were transferred to filters using a Brandell Cell Harvester (M24, Semat) which was washed through twice with distilled water and then with buffer, before suction of the cells onto a filter at 22mmHg vacuum pressure. Suction filtration removes any free ligand, leaving only bound [^{3}H]-spiperone on the filter. The advantage of this method is that it is rapid therefore preventing significant receptor-radioligand dissociation.

Once on the filter the samples were washed by drawing buffer over the filter twice for 5 sec to remove radioligand non-specifically bound to the filter and membrane, and dried for one hour in an LEEC drying oven, at full setting.

When dry, a Wallac Melt-on scintillation sheet was heat sealed to the filter, within a plastic cover, and the filter counted in a Wallac 1204 Betaplate BS liquid scintillation counter.

The disintegrations per minute (dpm) values obtained were converted into [^{3}H]-spiperone concentration, to give total binding, NSB, and free radioligand concentration. Therefore specific binding could be calculated (total - NSB) and plotted against free concentration to calculate B_{max} and K_D.

8.94 Competition Assay

Duplicate total binding tubes, duplicate NSB tubes and duplicate total counts (unfiltered) tubes were set up (4.9ml assay tubes, Sterilin), along with twelve competing drug concentration duplicates. A final assay concentration (F.A.C.) of 0.1nM [^{3}H]spiperone was used. The F.A.C. of the cold competitive ligand ranged from 1 x 10^{-6} to 3 x 10^{-12} M for spiperone, (+)butaclamol, quinperole and 7 OH-DPAT; and from 1 x 10^{-4} to 3 x 10^{-10} M for dopamine and (-)butaclamol. The drugs were weighed in powder form and then dissolved in ethanol and distilled water by vortexing and sonification to give 1mM solutions. Constituents were added in the order and quantities shown in Table 8.941.

The buffer used was the same as that in the saturation assay and the drug ketanserin was once again added at F.A.C. 1 x 10^{-7}M to block $5HT_2$ receptors.

Table 8.941 Competition Assay Composition

	total binding	NS binding	total counts	competition binding
Buffer	100	50	100	50
Buffer (10^{-7}M ketanserin)	100	100	100	100
[^{3}H]-spiperone	50	50	50	50
Membrane homogenate	250	250	250	250
Test drug	-	-	-	50
(+)butaclamol (10^{-6}M)	-	50	-	-
TOTAL VOLUME	500µl	500µl	500µl	500µl

The tubes were then vortexed and incubated at 37°C for 30 mins before being transferred to Whatman GF/B filters as described in the saturation assay method.

Dpm (disintegrations per minute) values were plotted against drug concentration and from the curve produced IC_{50}, and nH (Hill Coefficient) values were determined by a non-linear regression method. IC_{50} values for each cold competitor ligand were converted to pK_i to allow comparison of results. Competition assays were repeated three times (n=3) for each drug tested and mean and standard error values were calculated.

8.942 GTP Competition Assay

The competition assay was repeated for the agonist compounds (quinpirole, 7-hydroxy-DPAT and dopamine) using Gpp(NH)p, a non-hydrolysable GTP analogue. The competition assay method was repeated for each of the agonist ligands with the addition of Gpp(NH)p at a F.A.C. of 1 x 10^{-4}M.

8.95 Protein Assay Method

A Pierce BSA assay kit was used to determine the protein concentrations of the diluted membrane homogenate used in the assays.

Bovine serum albumin (BSA) at a stock concentration of 2mg/ml was serially diluted to F.A.C.s 1.0, 0.8, 0.6, 0.4, 0.2 and 0.1mg/ml. Then in 4.9ml Sterilin assay tubes, duplicate 50µl aliquots of these standard concentrations were assayed using the kit. Triplicate tubes of 50µl aliquots of the membrane homogenate were also assayed in this way.

After incubation the samples were measured for optical density (OD_{562}nm), and a standard curve plotted for the BSA assays (using buffer as zero). From the mean OD_{562} of the membrane samples a protein concentration could be calculated, using the curve, to give the milligrams of protein per millilitre of diluted homogenate and therefore the mg of protein per saturation assay tube. This was calculated using the Apple Macintosh application 'Cricket Graph' (Computer Associates).

APPENDIX I

DNA Miniprep Solutions

Cell Suspention Buffer

50mM Tris HCl, pH 7.5
10mM EDTA
100mg/ml RNase A

Cell Lysis Solution

0.2M NaOH(aq)
1% S.D.S.

Neutralization Solution

2.55M Potassium acetate, pH 4.8
60ml 5M CH_3COOK
11.5ml glacial acetic acid
28.5ml dH_2O

Column Wash Solution

200mM NaCl
20mM Tris HCl, pH 7.5
5mM EDTA
Dilute 1:1 with 95% ethanol.

Sequencing Solutions (Sequenase USB)

Enzyme Dilution Buffer

10mM Tris HCl, pH7.5
5mM DTT
0.5mg/ml Bovine Serum Albumin (BSA)

Primer Annealing Buffer

200mM Tris HCl, pH7.5
100mM $MgCl_2$
250mM NaCl

Labelling Mixture

1.5μM dTTP
1.5μM dGTP
1.5μM dCTP
1.5μM [α ^{35}S] dATP (1500 Ci/mmol)

Molecular Experimental Solutions

Tris [hydroymethyl] aminomethane hydrochloride (Tris HCl) (1M)

121.1g Tris base was dissolved in 800ml dH_2O and the pH adjusted using conc. HCl (pH 7.4 = 70ml; pH7.6 = 60ml; pH8.0 = 42ml). The solution was then autoclaved. N.B. The pH of this solution decreases with increasing temperature, keep at RT.

Tris / EDTA (T.E.) (1x stock solution)

10mM Tris HCl, pH7.5
1mM EDTA

Ligand Binding Buffer (pH7.4 @ 4°C)

50mM	Tris HCl	(33.5g HCl; 4.85g Tris base)
120mM	NaCl	(34.8g)
2mM	$CaCl_2$	(10ml)
1mM	Mg_2SO_4	(1.23g)
1mM	EDTA	(1.46g)

All components were dissolved in 4.5 litres dH_2O and Tris base added to adjust the pH to 7.4 then the volume was made up to 5litres.

Electrophoresis Buffers

T.A.E. (50x)

242g Tris base was dissolved in 57.1ml glacial acetic acid and 100ml 0.5M EDTA pH8

The working concentration of 1x contains 0.04M Tris - acetate and 0.001M EDTA.

T.B.E. (5x)

54g Tris base and 27.5g boric acid were dissolved in 20ml 0.5M EDTA pH8. The working solution of 0.5x contains 0.045M Tris - borate and 0.001M EDTA.

N.B. If the solution precipitates on storage it should be disguarded.

Termination Mixtures
80μM dGTP
80μM dATP
80μM dCTP
80μM dTTP
plus 8μM dideoxy-dNTP

Stop Solution
20mM EDTA
95% Formamide
0.05% Bromophenol Blue
0.05% Xylene Cyanol FF

Acrylamide Gel Solution
420g Urea (sequencing grade)
250ml dH_2O
240ml 5x TBE
40% acrylamide colution (19:1 bis, Severn Biotechnology)
Make up to 1l with dH_2O and filter through Whatman No. 3 paper.

Stock Solutions
Ethylenediaminetetraacetic acid (E.D.T.A.) (0.5M stock solution)
186.1g disodium EDTA.2H2O was dissolved in 800ml dH_2O at room temperature (RT) by vigorous stirring and the pH was adjusted to 8 (using approx. 20g NaOH pellets). The solution was then autoclaved on the standard cycle.

Sodium Dodecyl/Lauryl Sulphate (S.D.S.) (10% stock solution)
100g electrophoresis grade S.D.S. was dissolved in 900ml dH_2O by strring at 68oC. The a pH of 7.2 was obtained by adding conc. HCl dropwise and dH_2O added to 1l.

ATP (0.1M stock solution)
60mg ATP was dissolved in 0.8ml dH_2O and the pH adjusted to 7 by dropwise addition of 0.1M NaOH. The volume was increased to 1ml with dH_2O and the solution aliquoted and stored at -70°C.

dNTPs

Were provided as individual 100mM stocks at pH7 (Pharmacia)

Sample Loading Buffer

This was used to aid loading of the sample into an agarose gel well by adding colour, increasing the density and to indicate the distance moved by the sample. Bromophenol blue dye moves towards the anode with approximately the same velocity as linear ds DNA of 300bp length and Xylene Cyanol FF moves similarly with the velocity of linear ds DNA of 4kb length (in 0.5x TBE).

4g sucrose was dissolved in 6ml dH_2O along with 2.5mg bromophenol blue and 1mg Xylene cyanol FF. The volume was increased to 10ml with dH_2O and stored at RT.

Sodium Acetate (3M stock solution, pH7)

408.1g sodium ? .$3H_2O$ was dissolved in 800ml dH_2O and dilute acetic acid used to bring the pH to 7. The volume was increased to 1l with dH_2O and the solution autoclaved.

Ammonium acetate (10M stock solution)

770g ammonium acetate was dissolved in 800ml dH_2O and the volume made up to 1l before filter sterilization.

Dithiothreitol (DTT)

3.09g DTT was dissolved in 20ml 0.01M sodium acetate (pH5.2), this was filter sterilised and stored at -20°C in 1ml aliquots.

Magnesium Chloride ($MgCl_2$) (2M stock solution)

19g $MgCl_2$ was dissolved in 90ml dH_2O, then the volume was made up to 100ml and autoclaved.

Na45 Paper - Activation solution

The strip of paper was taken using forceps and soaked for 10mins in 10mM EDTA, then transferred to 0.5M NaOH for 5mins. It was then washed quickly in dH_2O before submerging in fresh dH_2O and storing at 4°C. It was cut into appropriately sized strips for use and if not used disguarded after 4 months.

Low salt NET (for use with Na45 paper)
100mM NaCl(aq)
0.1mM EDTA
20mM Tris-HCl, pH8

High salt NET (for use with Na45 paper)
1M NaCl
0.1mM EDTA
20mM Tris-HCl, pH8

Microbiological solutions (prepared and used aseptically)
Isopropylthio-b-D-galactoside (IPTG) MW 238.3 (powder at -20°C)
Stock solution at 100mM
0.024g dissolved in 1ml dH_20 at RT
Filter sterilize using a sterile 0.22 μm filter unit (Sartorius, Minisart NML).
Store at 4°C. Use 4mM (40μl) per ?mm diameter petri dish.

5-bromo-4-chloro-3-indoyl-b-D-galactopyranoside (X gal) MW 408.6
(Store as powder in dry and dark conditions at 4°C)
Stock solution at 50mg/ml
0.01g dissolved in 1ml di-methyl formamide (DMF)
Store away from light at -20°C

Liquid Media
LB
10g bacto-tryptone
5g bacto-yeast extract
10g NaCl
950ml dH_2O
This was shaken till dissolved and the pH adjusted to 7 by the addition of approximately 0.2ml 5M NaOH solution. The volume was increased to 1l using dH_2O and autoclaved on liquid cycle at 15lb/sq in for 20mins.

2xYT (Yeast Tryptone) Broth

16g bacto-tryptone
10g bacto-yeast extract
5g NaCl
900ml dH_2O

This solution was shaken until all solids were dissolved and the pH adjusted to 7 by the addition of approximately 0.2ml 5M NaOH. The volume was increased to 1l using dH_2O and autoclaved on liquid cycle at 15lb/sq in for 20mins.

SOC Media

20g bacto-tryptone
5g bacto-yeast extract
0.5g NaCl
950ml dH_2O

This solution was shaken until all solids were dissolved then 10ml 250mM KCl (1.86g KCl in 100ml dH_20) was added. The pH was adjusted to 7 by the addition of approximately 0.2ml 5M NaOH and the volume was increased to 1l using dH_2O and autoclaved on liquid cycle at 15lb/sq in for 20mins. On cooling to 60°C, 1M glucose (18g dissolved in 100ml dH_2O and filter sterilized through a 0.22 micron filter) was added to a final concentration of 20mM and 2M $MgCl_2$ (19g $MgCl_2$ dissolved in 100ml dH_2O and autoclaved) added to 10mM.

Solid Media

L Agar

Bacto-agar was added to LB media at 15g/l before autoclaving. This solid media could then be heated till molten when required for pouring into plates.

Antibiotics	**stock conc.**	**working conc.**
Ampicillin	50mg/ml in dH_2O	20 - 60mg/ml
Tetracyclin	5mg/ml in EtOH	10 - 50mg/ml
Kanamycin	10mg/ml in dH_2O	10 - 50mg/ml

APPENDIX II

Alignment of full length amino acid sequences for all neuropeptide receptors investigated in Chapter 3

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                1                                                   50
          PTH  ..........  ..........  ..........  ..........  GGGRGGELGG
    secretin  ..........  ..........  ........C*  QSCHLLQGAA  ASSLAVDLIG
  calcitonin  ..........  ..........  .......CAR  PHLTLRLTSP  AQEMGAEA*A
somatostatin  ..........  ..........  ..........  ..........  ..........
    d-opioid  ..........  ..........  ..........  ..........  ..........
rangiotensinII  ..........  ..........  ..........  ..........  ..........
         VIP  ..........  ..........  ..........  ..........  ..........
neuropeptide Y  ..........  ..........  ..........  ..........  ..........
bradykinin 3  ..........  ..........  ..........  ..........  ..........
N-formyl pep  ..........  ..........  ..........  ..........  ..........
         TRH  ..........  ..........  ..........  ..........  ..........
  neurotensin  ..........  ..........  ..........  ..........  ..........
     bombesin  ..........  ......NCSQ  RDSV*DGGRK  S*DKVGLILS  FSSG*VSVAC
   endothein  ..........  ..........  ..........  ..........  ..........
        NK-1  ..........  ..........  ..........  ..........  ..........
        NK-3  ..........  ..........  ..........  ....IAVSFS  FQSYLKTPAP
        NK-2  AHRLGTLCTS  VIWDVP*IAL  REI*IQIRVC  HQLPECLLLD  ASFL*LSAF*
         CCK  ..........  ..........  ..........  ..........  ..........
   Consensus  ----------  ----------  R----Q--R-  --L---L---  ASSLGVEA--

                51                                                  100
          PTH  RRRL......  ...PRGTRP*  AVAMGAARIA  PSLALLLCCP  VLSSAYALVD
    secretin  LGRS......  ...SHGLSGL  GGRPGSWTTC  RSLSWTQNRP  PSS*R....G
  calcitonin  PESLEPHLRT  PFASIELCPA  AQ*QNSRIND  SH*STHFCHP  RMQFSGEKIS
somatostatin  ..........  ..........  ..........  ..........  ..........
    d-opioid  ..........  ..........  ..........  ..........  ..........
  angiotensin  ..........  ..........  ..........  ..........  ........PA
         VIP  ..........  ..........  ..........  ..........  ..........
neuropeptide Y  ..........  ..........  ..........  ..........  ..........
   bradykinin  ..........  ..........  ......RRVS  STVGDGVAGS  R*PWIEGLPT
N-formyl pep  ..........  ..........  ..........  ..........  ..........
         TRH  ..........  .........E  FRRV*RNCRS  ED*ASAK*SS  CQTDWTRFLL
  neurotensin  ..........  ..........  ..........  ..........  .........M
     bombesin  *LSECTDVLL  AGGVKAGTEP  TSTN*ARVWN  TSVCVCVCVC  VCVCEFRVF*
   endothelin  .......KLR  TGHRTPSGAG  SSMQPPPSLC  GRALVALVLA  CGLSRIWGEE
        NK-1  ..........  ..........  ..........  ..........  ..........
        NK-3  K*PGGR....  ......EELQ  RSLVLGCPWV  SGRVRDCRPV  AMATLPAAET
        NK-2  LWCEREEPIQ  RAIRMPPALR  LEFEFSISKR  CLNSLIGTLL  PAQPDSFLPE
         CCK  ..........  ..........  ...PQCLPRC  SENGELKLPL  GMAAKPTPGN
   Consensus  L----R--L-  TG--RG-ELQ  GS-MLSRR--  SSV-RLLC-P  -MASR-RLP-

                101                                                 150
          PTH  ADDVFTKEEQ  IFLLHRAQAQ  CDKLLKEVLH  T.AANIMESD  KGWTPASTSG
    secretin  QNAKGSNSGR  SMLSTMRPRL  SLLLLRLLLL  TKAAHTVGVP  PRLCDVRRVL
  calcitonin  GQRDLQKSKM  RFTFTSRCLA  LFLLLNHPTP  ILPAFSNQTY  PTIEPKPFLY
somatostatin  ..........  .......DL*  AAWF...*TE  WKAAMEM...  .SSEQLNGSQ
    d-opioid  ..........  .....SKGWV  PAPRAHGGDG  HGGAMELVPS  ARAELQSSPL
  angiotensin  LGGT*RSARG  AGLIFDKLI*  NGWVFI.*IT  H*CHPRKSAP  GVFDIVFATN
         VIP  ..........  ..........  ..........  ..........  MDLHLFDYAE
neuropeptide Y  ..........  .......*QS  DAEGLSAPVA  TASGEPAVTM  EGISIYTSDN
   bradykinin  TQNRLSREKD  DPHSSLS*VQ  MHCSWKRPVL  LSVHEPMPTT  ASLGIEMFNI
N-formyl pep  ..........  ..........  ..........  ..........  .PRARPQLVN
         TRH  QDWKLGPIST  SSTRETGSVT  E*RGERTTAK  TNRQKGEGWK  DVLESPCQRS
  neurotensin  HLNSSVPQGT  PGEPDAQPFS  GPQSEMEAT.  ..........  ..........
     bombesin  REIKRLTQIS  EPN*QTFSA*  LKNPEVTKQH  LEGAFEERSF  EMAPNNCSHL
   endothelin  RGFPPDRA..  .......TPL  LQTAEIMTPP  TKTLWPKGSN  ASLARSLAPA
        NK-1  ..........  ..........  ..........  ..AALPAKM.  DNVLPVDSDL
        NK-3  WIDGGGGVGA  DAVNLTASLA  AGAATGAVET  GWLQLLDQA.  GNLSSSPSAL
        NK-2  L*AWPSGYTC  LLLNRPSPLK  STHRRGFLFC  GNSRRNCRAR  PCVPGPESVM
         CCK  L...PLPAPP  RQVAFGRPCD  H*RRETGMSH  SPARQHLVES  SRMDVVDSLL
   Consensus  --RKLSP-GT  DFL--T--L-  LA-LL--P-T  TKAALPM-AS  D-LEIVDSDL
```

```
                151                                                 200
PTH             KPRKEKASGK FYPESKENKD VPTGSRRRGR PCLPEWDNIV CWPLGAPGEV
secretin        LEERAHCLQQ LSKEKKGALG PETAS..... GCEGLWDNMS CWPSSAPART
calcitonin      VVGRKKMMDA QYK.CYDRMQ QLPAYQGEGP YCNRTWDGWL CWDDTPAGVL
d-opioid        VNLSDAFPSA FPSAGANASG SPGARSASSL .ALAIAITAL YSAVCAVGLL
angiotensin     STQVIKMILN SSTEDGIKRI QDDCPKAGRH NYIFVMIPTL YSIIFVVGIF
VIP             PGNFSDISWP CNSSDCIVVD TVMCPNMPNK SVLLYTLSFI YIFIFVIGMI
neuropeptide Y  YTE....EMG SGDYDSMK.. .EPCFREENA NFNKIFLPTI YSIIFLTGIV
bradykinin      TTQ....ALG SAHNGTFS.. EVNCPDTEWW SWLNAIQAPF LWVLFLLAAL
N-formyl peptide SPGADKMETN SSLPTNISGG TPAVSAG..Y LFLDIITYLV FAVTFVLGVL
TRH             FKPLKMENDT VSEMNQTELQ PQAAVALEYQ VVTILLVVII C....GLGIV
neurotensin     FLALSLSNGS GNTSESDTAG PNSDLDVNTD IYSKVLVTAI YLALFVVGTV
bombesin        NLDVDPFLSC N..DTFNQSL SPPKMDNWFH PGFIYVIPAV YGLIIVIGLI
endothelin      EVPKGDRTAG SPPRTISPP. .PCQGPIEIK ETFKYINTVV SCLVFVLGII
NK-1            .FPNTSTNTS E.....SNQ. ....FVQPT. .WQIVLWAAA YTVIVVTSVV
NK-3            GLPVASPAPS QPWANLTNQ. ....FVQPS. .WRIALWSLA YGVVVAVAVL
NK-2            GTRAIVSDAN ILSGLESNAT GVTAFSMPG. .WQLALWATA YLALVLVAVT
CCK             MNGSNITPPC EL.GLENETL FCLDQPQPSK EWQSALQILL YSIIFLLSVL
Consensus       -LPA-KMELN SS-EDL-NQG -P--FVQPR- SWLIVLIPLI Y-VIFVVGVL

                201                                                 250
PTH             VAVPCPDYIY DFNHK.GHAY RRCDRNGSWE VVPGHNRTWA NYSECLKFMT
secretin        VEVQCPKFLL MLSNKNGSLF RNCTQDGWSE TFP.....RP DLACGVNINN
calcitonin      SYQFCPDYFP DF.DPSEKVT KYCDEKGVWF KHPENNRTWS NYTMCNAFTP
somatostatin    GNTLV..IYV ILRYA...KM KTITNIYILN LAIADELFML GLPFLAMQVA
d-opioid        GNVLV..MFG IVRYT...KL KTATNIYIFN LALADALATS TLPFQSAKY.
angiotensin     GNSLV..VIV IYFYM...KL KTVASVFLLN LALADLCFLL TLPLWAVYTA
VIP             ANSVV..VWV NIQAK...TT GYDTHCYILN LAIADLWVVL TIPVWVVSLV
neuropeptide Y  GNGLV..ILV MGYQK...KL RSMTDKYRLH LSVADLLFVI TLPFWAVDAV
bradykinin      ENIFV..LSV FCLHK...TN CTVAEIYLGN LAAADLILAC GLPFWAITIA
N-formyl peptide GNGLV..IWV AGFRM...T. HTVTTISYLN LAVADFCFTS TLPFFMVRKA
TRH             GNIMV..VLV VMRTK...HM RTPTNCYLVS LAVADLMVLV AAGLPNITDS
neurotensin     GNSVT..AFT LARKKSLQSL QSTVHYHLGS LALSDLLILL LAMPVELYNF
bombesin        GNITL..IKI FCTVK...SM RNVPNLFISS LALGDLLLLV TCAPVDASKY
endothelin      GNSTL..LRI IYKNK...CM RNGPNILIAS LALGDLLHIV IDIPINVYKL
NK-1            GNVVV..IWI ILAHK...RM RTVTNYFLVN LAFAEACMAA FNTVVNFTYA
NK-3            GNLIV..IWI ILAHK...RM RTVTNYFLVN LAFSDASMAA FNTLVNFIYA
NK-2            GNATV..IWI ILAHE...RM RTVTNYFIIN LALADLCMAA FNATFNFIYA
CCK             GNTLV..ITV LIRNK...RM RTVTNIFLLS LAVSDLMLCL FCMPFNLIPN
Consensus       GNVLVPDIWV ILRHKSG--M RTVTNIFLLN LALADLLFLL TLPFWN-YYA

                251                                                 300
PTH             N.ETREREVF DRLGMIYTVG YSMSLASLTV AVLILAYFRR LH.........
secretin        SFNERRHAYL LKLKVMYTVG YSSSLAMLLV ALSILCSFRR LH.........
calcitonin      ..EKLKNAYV ..LYYLAIVG HSLSIFTLVI SLGIFVFFRK LTTIFPLNWK
somatostatin    L..V.HWPFG KAICRVVMTV DGINQFTSIF CLTVMSIDRY LAVVHPIKSA
d-opioid        L..METWPFG ELLCKAVLSI DYYNMFTSIF TLTMMSVDRY IAVCHPVKAL
angiotensin     M..EYRWPFG NYLCKIASAS VSFNLYASVF LLTCLSIDRY LAIVHPMKSR
VIP             Q..HNQWPMG ELTCKVTHLI FSINLFSGIF FLTCMSVDRY LSITYFTNTP
neuropeptide Y  A..N..WYFG NFLCKAVHVI YTVNLYSSVL ILAFISLDRY LAIVHATNSQ
bradykinin      N..NFDWLFG EVLCRVVNTM IYMNLYSSIC FLMLVSIDRY LALVKTMSMG
N-formyl peptide M..GGHWPFG WFLCKFLFTI VDINLFGSVF LIALIALDRC VCVLHPVWTQ
TRH             IYG..SWVYG YVGCLCITYL QYLGINASSC SITAFTIERY IAICHPIKAQ
neurotensin     IWVHHPWAFG DAGCRGYYFL RDACTYATAL NVASLSVERY LAICHPFKAK
bombesin        L..ADRWLFG RIGCKLIPFI QLTSVGVSVF TLTALSADRY KAIVRPMDIQ
endothelin      L..AEDWPFG AEMCKLVPFI QKASVGITVL SLCALSIDRY RAVASWSRIK
NK-1            V..HNVWYYG LFYCKFHNFF PIAALFASIY SMTAVAFDRY MAIIHPLQPR
NK-3            L..HSEWYFG ANYCRFQNFF PITAVFASIY SMTAIAVDRY MAIIDPLKPR
NK-2            S..HNIWYFG RAFCYFQNLF PITAMFVSIY SMTAIAADRY MAIVHPFQPR
CCK             L..LKDFIFG SAVCKTTTYF MGTSVSVSTF NLVAISLERY GAICRPLQSR
Consensus       L-EH-EWPFG EFLCKFVNFF P--NLFASIF SLTALS-DRY LAIVHPLKSR
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                301                                                 350
          PTH  .........CT RNYIHMHMFL SFMLRAASIF VKDAVLYS.. GFTLDEAERL
    secretin  .........CT RNYIHMHLFV SFILRALSNF IKDAVLFSSD DVTYCDAHKV
  calcitonin  YRKALSLGCQ RVTLHKNMFL TYILNSMIII IHLVEVVPNG ELVRRDP...
 somatostatin .........K WRRPRTAKMI NVAVWCVSLL VILPIMLYAG LR....SNQW
   d-opioid   .........D FRTPAKAKLI NICIWVLASG VGVPIMVMAV TQ....PRD.
angiotensin   .........L RRTMLVAKVT CIIIWLLAGL ASLFAIIHRN V..FFIENTN
        VIP   .........S SRKKMVRRVV CILVWLLAFC VSLPDTYYLK T..VTSASNN
neuropeptide Y .........R PRKLLAEKVV YVGVWIPALL LTIPDFIFAN V....SEADD
  bradykinin  .........R MRGVRWAKLY SLVIWSCTLL LSSPMLVFRT MKDYREEGHN
N-formyl peptide .........N HRTVSLAKKV IIGPWVMALL LTLPVIIRVT T....VPGKT
        TRH   .........F LCTFSRAKKI IIFVWAFTSI YCMLWFFLLD L....NISTY
 neurotensin  .........T LMSRSRTKKF ISAIWLASAL LAIPMLFTMG LQNRSGDGTH
    bombesin  .........A SHALMKICLK AALIWIVSML LAIPEAVFSD LHPFHVKDTN
  endothelin  .........G IGVPKWTAVE IVLIWVVSVV LAVPEAIGFD IITMDYKGSY
        NK-1  .........L SATATKV..V IFVIWVLALL LAFPQGYYS. ....TTETMP
        NK-3  .........L SATATKI..V IGSIWILAFL LAFPQCLYS. ....KTKVMP
        NK-2  .........L SAPSTKA..I IAGIWLVALA LASPQCFYS. ....TITVDE
         CCK  .........V WQTKSHALKV IAATWCLSFT IMTPYPIYSN LVPFTKNNNQ
   Consensus  ---------CL RRTLLKAK-V IIIIWVLALL LALPEL-YSD --T-TIE-TN

                351                                                 400
          PTH  TEEELHIIAQ VPPPPAAAAV GYAGCRVAVT FFLYFLATNY YWILVEGLYL
    secretin  .......... .......... ...GCKLVMI FFQYCIMANY AWLLVEGLYL
  calcitonin  .......... .......... ..VSCKILHF FHQYMMACNY FWMLCEGIYL
 somatostatin GRSSCTINWP GESG...... ....AWYTGF IIYAFILG.. .........FL
   d-opioid   GAVVCMLQFP SPSW...... ....YWDTVT KICVFLFA.. .........FV
angiotensin   ITVC.AFHYE SQNS...... ....TLPIGL GLTKNILG.. .........FL
        VIP   ETYCRSFYPE HSIK...... ....EWLIGM ELVSVVLG.. .........FA
neuropeptide Y RYICDRFY.. .PND...... ....LWVVVF QFQHIMVG.. .........LI
  bradykinin  VTACVIVY.. .PSR...... ....SWEVFT NMLLNLVG.. .........FL
N-formyl peptide GTVACTFNFS PWTNDPKERI NVAVAMLTVR GIIRFIIG.. .........FS
        TRH   KNAVVVSCGY KISRNY.... ....YSPIYL ..MDFGVF.. .........YV
 neurotensin  PGGLV..CTP IVDTAT.... ....VKVVIQ ..VNTFMS.. .........FL
    bombesin  QTFISCAPYP HSN..E.... ....LHPKIH SMASFLVF.. .........YV
  endothelin  LRICLLHPVQ KTAFMQ.... ....FYKTAK DWWLFSFY.. .........FC
        NK-1  SRVVCMIEWP EHPNRT.... ....YEKAYH ICVTVLIY.. .........F.
        NK-3  GRTLCFVQWP EGPKQH.... ....F..TYH IIVIILVY.. .........C.
        NK-2  GATKCVVAWP NDNGGK.... ....MLLLYH LVVFVLIY.. .........F.
         CCK  TANMCRFLLP SDAMQQ.... .......SWQ TFLLLILF.. .........L.
   Consensus  GRVCCMIYWP EP-RQT---- --AG-WP-YF IFV-FLVYNY -W-LVEGLFL

                401                                                 450
          PTH  HSLIFMAFFS EKKYLWGFTI FGWGLPAVFV AVWVGVRATL ANTGCWDLSS
    secretin  HTLLAISFFS ERKYLQAFVL LGWGSPAIFV ALWAITRHFL ENTGCWDINA
  calcitonin  HTLIVVAVFT EKQRLRWYYL LGWGFPLVPT TIHAITRAVY FNDNCW.LSV
 somatostatin VPLTIICLC. .......YLF IIIKVKSSGI RVGSSKRK.. ..........
   d-opioid   VPILIITVC. .......YGL MLLRLRSVRL LSGSKEKD.. ..........
angiotensin   FPFLIILTS. .......YTL IWKALKKAYE IQKNKPRN.. ..........
        VIP   VPFSIIAVF. .......YFL LARAISASSD QEKHSSR... ..........
neuropeptide Y LPGIVILSC. .......YCI IISKLSHSKG HQKRKA.... ..........
  bradykinin  LPLSII.TF. .......CTV RIMQVLRNNE MKKFKEVQ.. ..........
N-formyl peptide APMSIVAVS. .......YGL IATKIHKQGL IKSSRP.... ..........
        TRH   VPMILATVL. .......YGF IARILFLNPI PSDPKENSKM WKNDSIHQNK
 neurotensin  FPMLVISIL. .......NTV IANKLTV..M VHQAAEQGRV CTVGTHNGLE
    bombesin  IPLAIISVY. .......YYF IARNLIQSAY NLPVEGNIHV KK........
  endothelin  LPLAITAFF. .......YTL MTCEMLRKKS GMQIALNDHL KQ........
        NK-1  LPLLVIGYA. .......YTV VGITLWAS.. .......... .EIP.GDSSD
        NK-3  FPLLIMGIT. .......YTI VGITLWGG.. .......... .EIP.GDTCD
        NK-2  LPLLVMFGA. .......YSV IGLTLWKR.. .......... .AVPRHQAHG
         CCK  LPGIVMVVA. .......YGL ISLELYQGIK FDASQKKSAK EKKPSTGSST
   Consensus  LPLLIIAVFS EKKYL--YTL IG-TLWAS-- I-KSKERDHL K-IPCGDSSD
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                    451                                                 500
                PTH GHK.KWIIQV PILASVVLNF ILFINIIRVL ATKLRETNAG RCDTRQQYRK
          secretin NASVWWVIRG PVILSILINF IFFINILRIL MRKLR.TQET RGSETNHYKR
        calcitonin ETHLLYIIHG PVMAALVVNF FFLLNIVRVL VTKMRETHEA ...ESHMYLK
      somatostatin .......... .......... .......... .......... .....KSEKK
          d-opioid .......... .......... .......... .......... .....RSLRR
       angiotensin .......... .......... .......... .......... .....DDIFK
               VIP .......... .......... .......... .......... .........K
 neuropeptide Y    .......... .......... .......... .......... .........LK
        bradykinin .......... .......... .......... .......... .....TEKKA
 N-formyl peptide .......... .......... .......... .......... ..........
               TRH NLN....... .......... .......... ...LNATNRC FNSTVSSRKQ
       neurotensin HST....... .......... .......... ...FNMTIEP ..GRVQALRH
          bombesin .......... .......... .......... .......... ...QIESRKR
        endothelin .......... .......... .......... .......... ........RRE
              NK-1 RYHE...... .......... .......... .......... ...QVSAKRK
              NK-3 KYHE...... .......... .......... .......... ...QLKAKRK
              NK-2 ANLR...... .......... .......... .......... ...HLQAKKK
               CCK RYEDSDGCYL QKSRPPRKLE LQQLSSGSGG SRLNRIRSSS SAANLIAKKR
         Consensus RYHE-WII-G PV-AS-V-NF -FF-NI-RVL --KLRETNE- R-SQ--AK-K

                    501                                                 550
                PTH LLRSTLVLVP LFGVHYTVF. .......... .MALPYTEVS GTLWQIQMHY
          secretin LAKSTLLLIP LFGIHYIVF. .......... .AFSPEDAM. ....EVQLFF
        calcitonin AVKATMILVP LLGIQFVVF. .......... .PWRPSNKML GKIYDYVMH.
      somatostatin VTRMVSIVVA VFIFCWLPFY IFNVSSVSVA I.......SP TPALKGMFDF
          d-opioid ITRMVLVVVG AFVVCWAPIH IFVIVWTLVD IN.......RR DPLVVAALHL
       angiotensin II.MAIVL.. FFFFSWIPHQ IFTFLDVLIQ LGIIR.DCRI ADIVDTAMPI
               VIP II.FSYVV.. VFLVCWLPYH VAVLLDIFSI LHYIPFTCRL EHALFTALHV
 neuropeptide Y TT.VILIL.. AFFACWLPYY IGISIDSFIL LEIIKQGCEF ENTVHKWISI
        bradykinin TV.LVLAVLG LFVLCWFPFQ ISTFLDTLLR LGVLS.GCWN ERAVDIVTQI
 N-formyl peptide .LRVLSFVAA AFFLCWSPYQ VVALIATVRI RELLQGMYKE ...IGIAVDV
               TRH VTKMLAVVVI LFALLWMPYR TLVVVNSFLS SPFQEN..WF LLFCRIC...
       neurotensin GVLVLRAVVI AFVVCWLPYH VRRLMFCYIS DEQWTT..FL FDFYHYFYML
          bombesin LAKTVLVFVG LFAFCWLPNH VIYLYRSYHY SEVDTSM... ..LHFVTSIC
        endothelin VAKTVFCLVL VFALCWLPLH LSRILKLTLY NQNDPNRCEL LSFLLVLDYI
              NK-1 VVKMMIVVVC TFAICWLPFH IFFLLPYIN. .....PDLYL KKFIQQVYLA
              NK-3 VVKMMIIVVM TFAICWLPYH IYFILTAIY. .....QQLNR WKYIQQVYLA
              NK-2 FVKAMVLVVL TFAICWLPYH LYFILGTFQ. .....EDIYY HKFIQQVYLA
               CCK VIRMLIVIVV LFFLCWMPIF SANAWRAYD. .....TVSAE KHLSGTPISF
         Consensus VVKMM-VVV- LFAICWLP-H IFFLL--F-Y LE-IP-DC-L -KFIQIVYLI

                    551                                                 600
                PTH EMLFNSFQGF FVAIIYCFCN GEVQAEIRKS WSRWTLALDF KRKARSGSSS
          secretin ELALGSFQGL VVAVLYCFLN GEVQLEVQKK WRQWHLQ.EF PLRPVAFNNS
        calcitonin ..SLIHFQGF FVATIYCFCN NEVQTTVKRQ WAQFKIQWNQ RWGRRPSNRS
      somatostatin VVILTYANTC ANPILYAFLS DNFKKSFQNV LCLVKVSGTE DGERSDSKQD
          d-opioid CIALGYANSS LNPVLYAFLD ENFKRCFRQ. LCRTPCGRQE PGSLRRPRQA
       angiotensin TICIAYFNNC LNPLFYGFLG KKFKRYFLQL L...KYIPPK AKSHSN...L
               VIP TQCLSLVHCC VNPVLYSFIN RNYR...YEL M...KAFIFK YSAKTG...L
 neuropeptide Y TEALAFFHCC LNPILYAFLG AKFKTSAQHA L...TSVSRG SSLKIL...S
        bradykinin SSYVAYSNSC LNPLVYVIVG KRFRKKSREV Y...QAICRK GGCMGESVQM
 N-formyl peptide TSALAFFNSC LNPMLYVFMG QDFRERLIHA L......... ......PASL
               TRH ....IYLNSA INPVIYNLMS QKFRAAFRK. .......LCN CKQKPTEKAA
       neurotensin TNALFYVSSA INPILYNLVS ANFRQVFLST LAC....LCP GWRHRRKKRP
          bombesin AHLLAFTNSC VNPFALYLLS KSFRKQFNTQ L.LC...... CQPGLMNRSH
        endothelin GINMASLNSC INPIALYLVS KRFKNCFKSC L.CCWCQSFE EKQSLEEKQS
              NK-1 SMWLAMSSTM YNPIIYCCLN DRFRLGFKHA FRCCPFISAG DYEGLEMKST
              NK-3 SFWLAMSSTM YNPIIYCCLN KRFRAGFKRA FRWCPFIKVS SYDELELKTT
              NK-2 LFWLAMSSTM YNPIIYCCLN HRFRSGFRLA FRCCPWVTPT EEDRLELTHT
               CCK ILLLSYTSSC VNPIIYCFMN KRFRLGFMAT FPCCPNPGPP G.........
         Consensus T-WLAYFNSC LNPI-YCFLN KRFR-GF--A LRCCPFISP- DYD-LELKST
```

```
                601                                                   650
          PTH  YSYGPMVSHT SVTNV...GP RAGLSLPLSP RLPPATTNGH SQLPGHAKPG
    secretin  FSNATNGPTH STKA..... ....STEQSR SIPRA..... SII*
   calcitonin  ARAAAAAAEA GDIPIYICHQ EPRNEPANNQ GEESAEIIPL NIIEQESSA*
 somatostatin  KSRLNETTET QRTLLNGDLQ TSI*
     d-opioid  TTRERVTACT PSDGPGGGAA A*
  angiotensin  STKMSTLSYR PSDNVSSSTK KPAPCFEVE*
          VIP  .TKLIDAS.R VSETEYSALE QNAK*
neuropeptide Y  KGKRGGHSSV STESESSSFH SS*
    bradykinin  ENSMGTLRTS ISVDRQIHKL QDWAGNKQ*
N-formyl peptide  ERALTEDSTQ TSDTATNSTL PSAEVALQAK *
          TRH  NYSVALNYSV IKES..DRFS TELEDITVTD TYVSTTKVSF DDTCLASEN*
  neurotensin  TFSRKPN..S MSSN..HAFS TSATRETLY*
     bombesin  S..TGRSTTC MTSFKSTNPS ATFSLINRNI CHEGYV*
   endothelin  CLKFKANDHG YDNFRSSNKY SSS*
         NK-1  RYLQT.QSSV YKVSRLETTI STVVGAHEDE PEEGPKATPS SLDLTSNGSS
         NK-3  RFHPNRQSSM YTVTRMESMT VVFDPNDADT TRSSRKKRAT PRDPSFNGCS
         NK-2  PSLSRR.... ..VNRCHTKE TLFMTGDMTH SEATNGQVGS PQDGEPAGPI
          CCK  ..VRGEVGEE EDGRTIRALL SRYSYSHMST SAPPP*
    Consensus  -YKLT-QSS- YS--RS-S-- SSF--A---T -L-SRK--PS SQDPS-NGFS

                651                                                   700
          PTH  APATETETLP VTMAVPKDDG FLNGSCSG.. ..LD..EEAS GSARPPPLLQ
    secretin
   calcitonin
 somatostatin
     d-opioid
  angiotensin
          VIP
neuropeptide Y
    bradykinin
N-formyl peptide
          TRH
  neurotensin
     bombesin
   endothelin
         NK-1  RSNS....KT MTESSSFYSN ILA*
         NK-3  RRNS....KS ASATSSFISS PYT.....SV DEYS*
         NK-2  CKAQA*
          CCK
    Consensus  R--S----K- -T--SSF-S

                701
          PTH  EEWETVM*
```

All sequences obtained from GenEMBL database and translated using Wisconsin Genetics GCG program.

Database accession numbers:-

rat parathyroid hormone receptor (PTH) - *M77184*
rat secretin receptor - *X59132*
human calcitonin receptor - *L00587*
mouse somatostatin receptor - *M91000*
rat δ-opioid receptor - *L07271*
human angiotensin receptor - *M93394*
human neuropeptide Y receptor - *M84755*
rat bradykinin receptor - *X69681*
mouse thyrotropin releasing hormone receptor (TRH) - *M59811*
human vasoactive intestinal peptide receptor (VIP) - *L13288*
human N-formyl peptide receptor - *M60627*
rat cholecystokinin receptor (CCK) - *X01032*
rat neurotensin receptor - *P20789*
mouse bombesin receptor - *M61000*
human endothelinB receptor - *M74921*
mouse NK-1 receptor - *X62934*
human NK-3 receptor - *M89473*
rat NK-2 receptor - *M3183*

Alignment of full length amino acid coding sequences for the neuropeptide receptors in group i as assigned by peptide sequence homology in Table 2.46

```
            1                                                  50
       PTH  .......... ..GGGRGGEL GGRR..RLPR GTRP*AVAMG AARIAPSLAL
 secretin  C*QSCHLLQG AAASSLAVDL IGLG..RSSH GLSGLGGRPG SWTTCRSLSW
calcitonin GRTASPPDPG RAAPRLSRTF LGWRGARRPH LAQGLESPIQ EMRAGA*VPE
 Consensus ---------G -AA--L---L -G-R--R-PH G--GL----G --R---SL--

           51                                                 100
       PTH LLCCPVLSS. ..AYALVDAD DVFTKEEQIF LLHRAQAQCD KLLKEVLHT.
 secretin TQNRPPSS*. ..R....GQN AKGSNSGRSM LSTMRPRLSL LLLRLLLLTK
calcitonin SLEPPPANDL RFH*AVPGSP VRLLQD*QDH LQKSKMRFTL TRWCLTLFIF
 Consensus -L--PP-S-- ----A--G-- -------Q-- L-----R--L -LL-L-L-T-

           101                                                150
       PTH AANIMESDKG WIPASTSGKP RKEKASGKFY PESKENKDVP TGSRRRGRP.
 secretin AAHTVGVPPR LCDVRRVLLE ERAHCLQQLS KEKKGALGPE TAS.....G.
calcitonin LNRPLPVLPD SADGAHTPTL EPEPFLYILG KQRMLEAQHR CYDRMQKLPP
 Consensus AA----V-P- --D------- E-E--L--L- KE-K------ T-SR----P-

           151                                                200
       PTH .......CLP EWDNIVCWPL GAPGEVVAVP CPDYIYDFNH K.GHAYRRCD
 secretin .......CEG LWDNMSCWPS SAPARTVEVQ CPKFLLMLSN KNGSLFRNCT
calcitonin YQGEGLYCNR TWDGWSCWDD TPAGVLAEQY CPDYFPDF.D AAEKVTKYCG
 Consensus -------C-- -WDN-SCWP- -APG--VEV- CPDY--DF-- K-G---R-C-

           201                                                250
       PTH RNGSWEVVPG HNRTWANYSE CLKFMIN.ET REREVFDRLG MIYTVGYSMS
 secretin QDGWSETFP. ....RPDLAC GVNINNSFNE RRHAYLLKLK VMYTVGYSSS
calcitonin EDGDWYRHPE SNISWSNYTM C....NAFTP DKLQNAYILY YLAIVGHSLS
 Consensus -DG-WE--P- -N--W-NY-- C----N-F-- R-------L- --YTVGYS-S

           251                                                300
       PTH LASLTVAVLI LAYFRRLHCT RNYIHMHMFL SFMLRAASIF VKDAVLYS..
 secretin LAMLLVALSI LCSFRRLHCT RNYIHMHLFV SFILRALSNF IKDAVLFSSD
calcitonin ILTLLISLGI FMFLRSISCQ RVTLHKNMFL TYVLNSIIII VHLVVIVPNG
 Consensus LA-LLVAL-I L--FRRLHCT RNYIHMHMFL SF-LRA-SIF VKDAVL-S--

           301                                                350
       PTH GFTLDEAERL TEEELHIIAQ VPPPPAAAAV GYAGCRVAVT FFLYFLATNY
 secretin DVTYCDAHKV .......... .......... ...GCKLVMI FFQYCIMANY
calcitonin ELVKRDP... .......... .......... ..PICKVLHF FHQYMMSCNY
 Consensus --T--DA--- ---------- ---------- ---GCKV--- FFQY----NY

           351                                                400
       PTH YWILVEGLYL HSLIFMAFFS EKKYLWGFTI FGWGLPAVFV AVWVGVRATL
 secretin AWLLVEGLYL HTLLAISFFS ERKYLQAFVL LGWGSPAIFV ALWAITRHFL
calcitonin FWMLCEGVYL HTLIVVSVFA EGQRLWWYHV LGWGFPLIPT TAHAITRAVL
 Consensus -W-LVEGLYL HTLI--SFFS E-KYLW-F-- LGWG-PAIFV A-WAITRA-L

           401                                                450
       PTH ANTGCWDLSS GHK.KWIIQV PILASVVLNF ILFINIIRVL ATKLRETNAG
 secretin ENTGCWDINA NASVWWVIRG PVILSILINF IFFINILRIL MRKLR.TQET
calcitonin FNDNCW.LSV DTNLLYIIHG PVMAALVVNF FFLLNILRVL VKKLKESQEA
 Consensus -NTGCWDLS- -----WII-G PV-AS-V-NF IFFINILRVL --KLRETQE-

           451                                                500
       PTH RCDTRQQYRK LLRSTLVLVP LFGVHYTVFM ALPYTEVSGT LWQIQMHYEM
 secretin RGSETNHYKR LAKSTLLLIP LFGIHYIVFA FSPEDAM... ..EVQLFFEL
calcitonin ...ESHMYLK AVRATLILVP LLGVQFVVLP WRPSTPLLGK IYDYVVH...
 Consensus R--E---Y-K L-RSTL-LVP LFGVHY-VF- --P-T---G- ----Q-H-E-

           501                                                550
       PTH LFNSFQGFFV AIIYCFCNGE VQAEIRKSWS RWTLALDFKR KARSGSSSYS
 secretin ALGSFQGLVV AVLYCFLNGE VQLEVQKKWR QWHLQ.EFPL RPVAFNNSFS
calcitonin SLIHFQGFFV AIIYCFCNHE VQGALKRQWN QYQAQ....R WAGRRSTRAA
 Consensus -L-SFQGFFV AIIYCFCNGE VQ-E--K-W- QW-LQ--F-R -A---S-S-S
```

```
            551                                                   600
       PTH  YGPMVSHTSV TNVGPRAGLS LPLSPRLPPA TTNGHSQLPG HAKPGAPATE
 secretin   NATNGPTHST KA.......S TEQSRSIPRA .....SII*G CSK..APADE
calcitonin  NAAAATAAAA AALAETVEIP VYICHQEPRE EPAGEEPVVE VEGVEVIAME
 Consensus  NA------S- -A-------S ---S---PRA ---G-S---G --K--APA-E

            601                                                   650
       PTH  TETLPVTMAV PKDDGFLNGS CSGLD..EEA SGSARPPPLL QEEWETVM*L
 secretin   GLDI...... .R*DGHLSDS HLVLDWLNRL VSRVRASSEI KQEETT....
calcitonin  VLEQETSA*T *RKYSIVITE SSLPGRKTNL ASKM..ISIL PGGNISFVRI
 Consensus  -L-------- -R-DG-L--S -S-LD----L -S--R--S-L --E--T----

            651                                                   700
       PTH  GTRGLDCWPG HMDRWTKKPV FGWLSIGTGP GR*PKENGSG RSREEGRGFA
 secretin   .......RSS RSRKRTHDLV F.LVLLRRAI QQLQKGQG.. ..........
calcitonin  IQ*IFPS... ....*T*SKF YLVLLLRETV SEWSLPLQPL *TPVNSIFHP
 Consensus  ---------- -----T---V F--LLLR--- ----K--G-- ----------
```

Alignment of full length amino acid coding sequences for the neuropeptide receptors in group ii as assigned by peptide sequence homology in Table 2.46

```
           1                                                  50
     NK1   .......... .......... .......... .......... ..........
     NK3   .......... .......... .......... ....IAVSFS FQSYLKTPAP
     NK2   AHRLGTLCTS VIWDVP*IAL REI*IQIRVC HQLPECLLLD ASFL*LSAF*
Consensus  ---------- ---------- ---------- ---------- ----------

           51                                                 100
     NK1   .......... .......... .......... .......... ..........
     NK3   K*PGGR.... ......EELQ RSLVLGCPWV SGRVRDCRPV AMATLPAAET
     NK2   LWCEREEPIQ RAIRMPPALR LEFEFSISKR CLNSLIGTLL PAQPDSFLPE
Consensus  ---------- ---------- ---------- ---------- ----------

           101                                                150
     NK1   .......... .......... .......... ..AALPAKM. DNVLPVDSDL
     NK3   WIDGGGGVGA DAVNLTASLA AGAATGAVET GWLQLLDQA. GNLSSSPSAL
     NK2   L*AWPSGYTC LLLNRPSPLK STHRRGFLFC GNSRRNCRAR PCVPGPESVM
Consensus  ------G--- ---N----L- -----G---- G---L---A- -NV----S-L

           151                                                200
     NK1   .FPNTSTNTS E.....SNQ. ....FVQPTW QIVLWAAAYT VIVVTSVVGN
     NK3   GLPVASPAPS QPWANLTNQ. ....FVQPSW RIALWSLAYG VVVAVAVLGN
     NK2   GTRAIVSDAN ILSGLESNAT GVTAFSMPGW QLALWATAYL ALVLVAVTGN
Consensus  G-P--S---S ------SNQ- ----FVQP-W QIALWA-AY- V-V-VAV-GN

           201                                                250
     NK1   VVVIWIILAH KRMRTVTNYF LVNLAFAEAC MAAFNTVVNF TYAVHNVWYY
     NK3   LIVIWIILAH KRMRTVTNYF LVNLAFSDAS MAAFNTLVNF IYALHSEWYF
     NK2   ATVIWIILAH ERMRTVTNYF IINLALADLC MAAFNATFNF IYASHNIWYF
Consensus  --VIWIILAH KRMRTVTNYF LVNLAFADAC MAAFNT-VNF IYA-HN-WYF

           251                                                300
     NK1   GLFYCKFHNF FPIAALFASI YSMTAVAFDR YMAIIHPLQP RLSATATKVV
     NK3   GANYCRFQNF FPITAVFASI YSMTAIAVDR YMAIIDPLKP RLSATATKIV
     NK2   GRAFCYFQNL FPITAMFVSI YSMTAIAADR YMAIVHPFQP RLSAPSTKAI
Consensus  G--YC-FQNF FPITA-FASI YSMTAIA-DR YMAIIHPLQP RLSATATK-V

           301                                                350
     NK1   IFVIWVLALL LAFPQGYYST TETMPSRVVC MIEWPEHPNR TYEKAYHICV
     NK3   IGSIWILAFL LAFPQCLYSK TKVMPGRTLC FVQWPEGPKQ HF..TYHIIV
     NK2   IAGIWLVALA LASPQCFYST ITVDEGATKC VVAWPNDNGG KMLLLYHLVV
Consensus  I--IW-LALL LAFPQC-YST T-VMPGRT-C -V-WPE-P-- -----YHI-V

           351                                                400
     NK1   TVLIYFLPLL VIGYAYTVVG ITLWASEIP. GDSSDRYHEQ VSAKRKVVKM
     NK3   IILVYCFPLL IMGITYTIVG ITLWGGEIP. GDTCDKYHEQ LKAKRKVVKM
     NK2   FVLIYFLPLL VMFGAYSVIG LTLWKRAVPR HQAHGANLRH LQAKKKFVKA
Consensus  -VLIYFLPLL VMG-AYTVVG ITLW--EIP- GD--D-YHEQ L-AKRKVVKM

           401                                                450
     NK1   MIVVVCTFAI CWLPFHIFFL LPYINPDLYL KKFIQQVYLA SMWLAMSSTM
     NK3   MIIVVMTFAI CWLPYHIYFI LTAIYQQLNR WKYIQQVYLA SFWLAMSSTM
     NK2   MVLVVLTFAI CWLPYHLYFI LGTFQEDIYY HKFIQQVYLA LFWLAMSSTM
Consensus  MI-VV-TFAI CWLPYHIYFI L--I--DLY- -KFIQQVYLA SFWLAMSSTM

           451                                                500
     NK1   YNPIIYCCLN DRFRLGFKHA FRCCPFISAG DYEGLEMKST RYLQT.QSSV
     NK3   YNPIIYCCLN KRFRAGFKRA FRWCPFIKVS SYDELELKTT RFHPNRQSSM
     NK2   YNPIIYCCLN HRFRSGFRLA FRCCPWVTPT EEDRLELTHT PSLSRR....
Consensus  YNPIIYCCLN -RFR-GFK-A FRCCPFI--- -YD-LELK-T R-L--RQSS-

           501                                                550
     NK1   YKVSRLETTI STVVGAHEDE PEEGPKATPS SLDLTSNGSS RSNS....KT
     NK3   YTVTRMESMT VVFDPNDADT TRSSRKKRAT PRDPSFNGCS RRNS....KS
     NK2   ..VNRCHTKE TLFMTGDMTH SEATNGQVGS PQDGEPAGPI CKAQA*PL*E
Consensus  Y-V-R-ET-- --F---D-D- -E---K---S P-D---NG-S R-NS----K-
```

```
           551                                                    600
     NK1   MTESSSFYSN ILA*AARHNM .......... .......... ..........
     NK3   ASATSSFISS PYT.....SV DEYS*FHFLR *KI..... SVRPSWCQS.
     NK2   AADGKAPT** LVPHPILQHT NRKNRMVLGM PQAIQVSTKN SEHRHLSQAI
Consensus  A---SSF-S- ---------- ---------- ---------- S------Q--
```

Alignment of full length amino acid coding sequences for the neuropeptide receptors in group iii as assigned by peptide sequence homology in Table 2.46

```
                1                                                  50
          TRH  EFRRV*RNCR SED*ASAK*S SCQTDWTRFL LQDWKLGPIS TSSTRETGSV
  neurotensin  .......... .......... .......... MHLNSSVPQG TPGEPDAQPF
neuropeptide Y .......... .......... .......... .......... ..*QSDAEGL
    Consensus  ---------- ---------- ---------- -------P-- T----DA---

               51                                                 100
          TRH  TE*RGERTTA KTNRQKGEGW KDVLESPCQR SFKPLKMEND TVSEMNQTEL
  neurotensin  SGPQSEMEAT .......... .......... .FLALSLSNG SGNTSESDTA
neuropeptide Y SAPVATASGE PAVTMEG... .......... ..ISIYTSDN YTEEMGSGDY
    Consensus  S-P--E---- ------G--- ---------- -F--L--SN- ---EM-S---

              101                                                 150
          TRH  QP..QAAVAL EYQVVTILLV VIIC....GL GIVGNIMVVL VVMRTK...H
  neurotensin  GP..NSDLDV NTDIYSKVLV TAIYLALFVV GTVGNSVTAF TLARKKSLQS
neuropeptide Y DSMKEPCFRE ENANFNKIFL PTIYSIIFLT GIVGNGLVIL VMGYQK...K
    Consensus  -P-------- E-----K-LV --IY---F-- GIVGN--V-L V--R-K----

              151                                                 200
          TRH  MRTPTNCYLV SLAVADLMVL VAAGLPNITD SIYG..SWVY GYVGCLCITY
  neurotensin  LQSTVHYHLG SLALSDLLIL LLAMPVELYN FIWVHHPWAF GDAGCRGYYF
neuropeptide Y LRSMTDKYRL HLSVADLLFV ITLPFWAVDA VA....NWYF GNFLCKAVHV
    Consensus  LRS-T--YL- SLAVADLL-L --A------- -I-----W-F G--GC-----

              201                                                 250
          TRH  LQYLGINASS CSITAFTIER YIAICHPIKA QFLCTFSRAK KIIIFVWAFT
  neurotensin  LRDACTYATA LNVASLSVER YLAICHPFKA KTLMSRSRTK KFISAIWLAS
neuropeptide Y IYTVNLYSSV LILAFISLDR YLAIVHATNS Q......RPR KLLAEKVVYV
    Consensus  L-----YAS- L--A--S-ER YLAICHP-KA Q-L---SR-K K-I---W---

              251                                                 300
          TRH  SIYCMLWFFL LDL....NIS TYKNAVVVSC GYKISRNYYS PIYLMD.FGV
  neurotensin  ALLAIPMLFT MGLQNRSGDG THPGGLV..C TPIVDTATVK VVIQVN.TFM
neuropeptide Y GVWIPALLLT IPDFIFANVS EADDRYI..C DRFYPNDLWV VVFQFQHIMV
    Consensus  -------LFT --L----N-S T-----V--C ---------- VV-Q-----V

              301                                                 350
          TRH  FYVVPMILAT VLYGFIARIL FLNPIPSDPK ENSKMWKNDS IHQNKNLNLN
  neurotensin  SFLFPMLVIS ILNTVIANKL TV..MVHQAA EQGRVCTVGT HNGLEHSTFN
neuropeptide Y GLILPGIVIL SCYCIIISKL S......... .......... ..........
    Consensus  ----PMIVI- -LY--IA-KL ---------- E--------- ---------N

              351                                                 400
          TRH  ATNRCFNSTV SSRKQVTKML AVVVILFALL WMPYRTLVVV NSFLSSPF..
  neurotensin  MTIEP..GRV QALRHGVLVL RAVVIAFVVC WLPYHVRRLM FCYISDEQ..
neuropeptide Y ......HSKG HQKRKALKTT VILILAFFAC WLPYYIGISI DSFILLEIIK
    Consensus  -T-----S-V ---R---K-L --VVIAF--C WLPY------ -SFIS-E---

              401                                                 450
          TRH  QENWFLLFCR IC.......I YLNSAINPVI YNLMSQKFRA AFRK....LC
  neurotensin  WTTFLFDFYH YFYMLTNALF YVSSAINPIL YNLVSANFRQ VFLSTLACLC
neuropeptide Y QGCEFENTVH KWISITEALA FFHCCLNPIL YAFLGAKFKT SAQHALTSVS
    Consensus  Q---F--F-H -----T-AL- Y--SAINPIL YNL-SAKFR- -F---L--LC

              451                                                 500
          TRH  NCKQKPTEKA ANYSVALNYS VIKESDRFST ELEDIT.... ..........
  neurotensin  PGWRHRRKKR PTFSRKPN.. SMSSNHAFST SATRET*... ..........
neuropeptide Y RGSSLKILSK GKRGGHSSVS TESESSSFHS S*HRCKRLFF IR*ITFF*VT
    Consensus  -G------K- ---S---N-S --SES--FST S--R-T---- ----------

              501                                                 550
          TRH  .......... .......... ..VTDTYVST TKVSFDDTCL ASEN*RRP*R
  neurotensin  .......... .......... .......... .......... ..........
neuropeptide Y HFSDIKD*PI LYSFYCLLDF CLVFL*FL*S LIDLFI*IFF VSY*CVSRQD
    Consensus  ---------- ---------- --V------- ----F----- -S--------
```

Alignment of full length amino acid coding sequences for the neuropeptide receptors in group iv as assigned by peptide sequence homology in Table 2.46

```
            1                                                  50
 bombesin   NCSQRDSV*D GGRKS*DKVG LILSFSSG*V SVAC*LSECT DVLLAGGVKA
endothlin   .......... .......... .......... .......... .KLRTGHRTP
      CCK   .......... .......... .......... .......... ..........
Consensus   ---------- ---------- ---------- ---------- --L--G----

            51                                                 100
 bombesin   GTEPTSTN*A RVWNTSVCVC VCVCVCVCEF RVF*REIKRL TQISEPN*QT
endothlin   SGAGSSMQPP PSLCGRALVA LVLACGLSRI WGEERGFPPD RA........
      CCK   ....PQCLPR CSENGELKLP LGMAAKPTPG NLPLPAPPRQ VAFGRP....
Consensus   -----S--P- -S-NG---V- L--A------ --FLR--PR- -A---P----

            101                                                150
 bombesin   FSA*LKNPEV TKQHLEGAFE ERSFEMAPNN CSHLNLDVDP FLSCN..DTF
endothlin   .TPLLQTAEI MTPPTKTLWP KGSNASLARS LAPAEVPKGD RTAGSPPRTI
      CCK   CDH*RRETGM SHSPARQHLV ESSRMDVVDS LLMNGSNITP PCELGLENET
Consensus   ----L---E- ---P------ E-S------S L---------P --------T-

            151                                                200
 bombesin   NQSLSPPKMD NWFHPGFIYV IPAVYGLIIV IGLIGNITLI KIFCTVKSMR
endothlin   SPP..PCQGP IEIKETFKYI NTVVSCLVFV LGIIGNSTLL RIIYKNKCMR
      CCK   LFCLDQPQPS KEWQSALQIL ...LYSIIFL LSVLGNTLVI TVLIRNKRMR
Consensus   ---L-PPQ-- -E----F-Y- ---VY-LIFV LG-IGN-TLI -I---NK-MR

            201                                                250
 bombesin   NVPNLFISSL ALGDLLLLVT CAPVDASKYL ADRWLFGRIG CKLIPFIQLT
endothlin   NGPNILIASL ALGDLLHIVI DIPINVYKLL AEDWPFGAEM CKLVPFIQKA
      CCK   TVTNIFLLSL AVSDLMLCLF CMPFNLIPNL LKDFIFGSAV CKTITTYFMGT
Consensus   NVPNIFI-SL ALGDLLL-V- C-P----K-L A-DW-FG--- CKL-PFIQ-T

            251                                                300
 bombesin   SVGVSVFTLT ALSADRYKAI VRPMDIQASH ALMKICLKAA LIWIVSMLLA
endothlin   SVGITVLSLC ALSIDRYRAV ASWSRIKGIG VPKWTAVEIV LIWVVSVVLA
      CCK   SVSVSTFNLV AISLERYGAI CRPLQSRVWQ TKSHALKVIA ATWCLSFTIM
Consensus   SVGVSVF-L- ALS-DRY-AI -RP--I---- --------IA LIW-VS--LA

            301                                                350
 bombesin   IPEAVFSDLH PFHVKDTNQT FISCAPYPHS N..ELHPKIH SMASFLVFYV
endothlin   VPEAIGFDII TMDYKGSYLR ICLLHPVQKT AFMQFYKTAK DWWLFSFYFC
      CCK   TPYPIYSNLV PFTKNNNQTA NMCRFLLPSD AMQQSWQT.. ..FLLLILFL
Consensus   -PEAI-SDL- PF--K----- -----P---- A--Q------ ---LFL--F-

            351                                                400
 bombesin   IPLAIISVYY YFIARNLIQ. .......... .......... ..........
endothlin   LPLAITAFFY TLMTCEMLR. .......... .......... ..........
      CCK   LPGIVMVVAY GLISLELYQG IKFDASQKKS AKEKKPSTGS STRYEDSDGC
Consensus   LPLAI--V-Y -LI--EL-Q- ---------- ---------- ----------

            401                                                450
 bombesin   .......... .......... SAYNLPVEGN IHVKKQIESR KRLAKTVLVF
endothlin   .......... .......... KKSGMQIALN DHLKQ....R REVAKTVFCL
      CCK   YLQKSRPPRK LELQQLSSGS GGSRLNRIRS SSSAANLIAK KRVIRMLIVI
Consensus   ---------- ---------- --S-L----N -H-K-----R KRVAKTV-V-

            451                                                500
 bombesin   VGLFAFCWLP NHVIYLYRSY HYSEVDTSM. ....LHFVTS ICAHLLAFTN
endothlin   VLVFALCWLP LHLSRILKLT LYNQNDPNRC ELLSFLLVLD YIGINMASLN
      CCK   VVLFFLCWMP IFSANAWRAY DTVSAEKHLS GTPISFILL. .....LSYTS
Consensus   V-LFA-CWLP -H-----R-Y -Y---D---- -------VL- -----LAFTN

            501                                                550
 bombesin   SCVNPFALYL LSKSFRKQFN TQLLC..... .....CQPGL MNRSHS..TG
endothlin   SCINPIALYL VSKRFKNCFK SCLCC....W CQSFEEKQSL EEKQSCLKFK
      CCK   SCVNPIIYCF MNKRFRLGFM ATFPCCPNPG PPGVRGEVGE EEDGRTIRAL
Consensus   SCVNPIALYL -SKRFR--F- --L-C----- --------GL EE--------
```

```
          551                                                        600
bombesin  RSTTCMTSFK STNPSATFSL INRNICHEGY V*TKLQPCL* RNSWYCSTDV
endothlin ANDHGYDNFR SSNKYSSS*K KNYSLYFIFF ILDRSH*NKM KHLPKQNKKL
      CCK LSRYSYSHMS TSAPPP*TPP GPL..... .......... ..........
Consensus -S---Y--F- SSNP------ -N-------- ---------- ----------
```

Alignment of full length amino acid coding sequences for the neuropeptide receptors in group v as assigned by peptide sequence homology in Table 2.46

```
                    1                                                  50
      angiotensin   .......... .......... ........PA LGGT*RSARG AGLIFDKLI*
        bradykinin  ......RRVS STVGDGVAGS R*PWTEGLPT TQNRLSREKD DPHSSLS*VQ
n-formyl peptide    .......... .......... .......... .......... ..........
        Consensus   -------R-- --V------- -----E--PA L----S---- ---I----I-

                    51                                                 100
      angiotensin   NGWVFI.*IT H*CHPRKSAP GVFDIVFAIN STQVIKMILN SSTEDGIKRI
        bradykinin  MHCSWKRPVL LSVHEPMPTT ASLGIEMFNI TTQ....ALG SAHNGTFSEV
n-formyl peptide    .......... .......... .PRARPQLVN SPGADKMETN SSLPTNIS..
        Consensus   M--------IT ---H------ --F-IV--TN STQ--KMALN SS-EDGI-RI

                    101                                                150
      angiotensin   QDDCPKAGRH NYIFVMIPTL ..YSIIFVVG IFGNSLVVIV IYFYMKLKTV
        bradykinin  ..NCPDTEWW SWLNAIQAPF ..LWVLFLLA ALENIFVLSV FCLHKTNCTV
n-formyl peptide    ..GGTPAVSA GYLFLDIITY LVFAVTFVLG VLGNGLVIWV AGFRMT.HTV
        Consensus   QDDCPKAGRH SY-FVMIPTL --YS-IFV-G I-GNSLVVIV IYFYM-LKTV

                    151                                                200
      angiotensin   ASVFLLNLAL ADLCFLLTLP LWAVYTAMEY RWPFGNYLCK IASASVSFNL
        bradykinin  AEIYLGNLAA ADLILACGLP FWAITIANNF DWLFGEVLCR VVNTMIYMNL
n-formyl peptide    TTISYLNLAV ADFCFTSTLP FFMVRKAMGG HWPFGWFLCK FLFTIVDINL
        Consensus   AS-FLLNLAL ADLCFLLTLP -WAVYTAMEY RWPFGN-LCK IAS-SV-FNL

                    201                                                250
      angiotensin   YASVFLLTCL SIDRYLAIVH PMKSRLRRTM LVAKVTCIII WLLAGLASLP
        bradykinin  YSSICFLMLV SIDRYLALVK TMSMGRMRGV RWAKLYSLVI WSCTLLLSSP
n-formyl peptide    FGSVFLIALI ALDRCVCVLH PVWTQNHRTV SLAKKVIIGP WVMALLLTLP
        Consensus   YASVFLLT-- SIDRYLAIVH PMKSRLRRT- LVAKVTCIII WLMA-L-SLP

                    251                                                300
      angiotensin   AIIHRNV..F FIENTNITVC AFHYESQNST .......... LPIGLGLTKN
        bradykinin  MLVFRTMKDY REEGHNVTAC VIVYPSRSWE .......... VFTNMLL..N
n-formyl peptide    VIIRVTTVP. ..GKTGTVAC TFNFSPWTND PKERINVAVA MLTVRGIIRF
        Consensus   AIIHR-V--Y FIENTNIT-C AFHYESRNS- ---------- L--GLGLTKN

                    301                                                350
      angiotensin   ILGFLFPFLI ILTSYTLIWK ALKKAYEIQK NKPRNDD..I FKIIMAIVLF
        bradykinin  LVGFLLPLSI I.TFCTVRIM QVLRNNEMKK FKEVQTEKKA TVLVLAVLGL
n-formyl peptide    IIGFSAPMSI VAVSYGLIAT KIHKQGLIKS SRP....... LRVLSFVAAA
        Consensus   I-GFLFPF-I ILTSYTLIWK A-KKAYEI-K NKPRNDD--I FRIIMA-VLF

                    351                                                400
      angiotensin   FFFSWIPHQI FTFLDVLIQL GIIRDCRIAD IVDTAMPITI CIAYFNNCLN
        bradykinin  FVLCWFPFQI STFLDTLLRL GVLSGCWNER AVDIVTQISS YVAYSNSCLN
n-formyl peptide    FFLCWSPYQV VALIATVRIR ELLQG..MYK EIGIAVDVTS ALAFFNSCLN
        Consensus   FF--W-P-QI FTFLD-LIQL GV---C-I-D IVD-AMPIT- CIAYFN-CLN

                    401                                                450
      angiotensin   PLFYGFLGKK FKRYFLQLLK YIPPKA.KSH SNLSTKMSTL SYRPSDN...
        bradykinin  PLVYVIVGKR FRKKSREVYQ AICRKG.GCM GE........ ....SVQ...
n-formyl peptide    PMLYVFMGQD FRERLIHALP ASLERALTED STQTSDTATN STLPSAEVAL
        Consensus   PLFY-FLGKK F-KYFLQLLK -IPPKA-KSH S-LSTKMSTL SYRPSDN---

                    451                                                500
      angiotensin   .......... VSSSTKKPAP CFEVE*HVRN LSIK*FCERR SKRTFLCSTS
        bradykinin  .......... MENSMGTLRT SISVDRQIHK LQ.DWAGNKQ *TQAIGQDDC
n-formyl peptide    QAK*GGSWGT LSSSQLQLRL TLS*AEHRHF LLILGLPTHQ KKKKSLCVP*
        Consensus   ---------- MSSS-KK--- CF-VE--VH- L-I---C--- SK-A-LCSD-
```

Alignment of full length amino acid coding sequences for the neuropeptide receptors in group vi as assigned by peptide sequence homology in Table 2.46

```
            1                                                       50
somatostatin ..........  ..........  .......DL*  AAWF...*TE  WKAAMEM...
    d-opioid ..........  ..........  .....SKGWV  PAPRAHGGDG  HGGAMELVPS
         VIP ..........  ..........  ..........  ..........  ....MDLHLF
Consensus    ----------  ----------  ----------  -A--------  ---AMEL---

            51                                                      100
somatostatin .SSEQLNGSQ  VWVSSPFDLN  GSLGPSNGSN  ..QTEPYYDM  TSNAVL....
   d-opioid  ARAELQSSPL  VNLSDAFPSA  FPSAGANASG  SPGARSASSL  .ALAIA...I
         VIP  DYAEPGNFSD  ISWPC.....  ....NSSDCI  VVDTVMCPNM  PNKSVLLYTL
  Consensus  --AE--N-S-  V--S--F---  -----SN-S-  ---------M  ---AVL----

            101                                                     150
somatostatin TFIYFVVCVV  GLCGNTLVIY  VILRYAKMKT  ITNIYILNLA  IADELFMLGL
   d-opioid  TALYSAVCAV  GLLGNVLVMF  GIVRYTKLKT  ATNIYIFNLA  LADALATSTL
         VIP  SFIYIFIFVI  GMIANSVVVW  VNIQAKTTGY  DTHCYILNLA  IADLWVVLTI
  Consensus  TFIY--VCVV  GL-GN-LV--  VI-RY-K-KT  -TNIYILNLA  IAD-L--LTL

            151                                                     200
somatostatin PFLAMQVALV  .HWPFGKAIC  RVVMTVDGIN  QFTSIFCLTV  MSIDRYLAVV
   d-opioid  PFQSAKY.LM  ETWPFGELLC  KAVLSIDYYN  MFTSIFTLTM  MSVDRYIAVC
         VIP  PVWVVSLVQH  NQWPMGELTC  KVTHLIFSIN  LFSGIFFLTC  MSVDRYLSIT
  Consensus  PF------L-  --WPFGEL-C  KVV--ID-IN  -FTSIF-LT-  MSVDRYLAV-

            201                                                     250
somatostatin HPIKSAKWRR  PRTAKMINVA  VWCVSLLVIL  PIMLYAGLRS  NQWGRSSCTI
   d-opioid  HPVKALDFRT  PAKAKLINIC  IWVLASGVGV  PIMVMAVTQP  RD.GAVVCML
         VIP  YFTNTPSSRK  KMVRRVVCIL  VWLLAFCVSL  PDTYYLKTVT  SASNNETYCR
  Consensus  HP-K----R-  P--AK-INI-  VW-LA--V-L  PIM-YA-T--  ---G---C--

            251                                                     300
somatostatin NWPGESGA..  WYTGFIIYAF  ILGFLVPLTI  ICLCYLFIII  KVKSSGIRVG
   d-opioid  QFPSPSWY..  WDTVTKICVF  LFAFVVPILI  ITVCYGLMLL  RLRSVRLLSG
         VIP  SFYPEHSIKE  WLIGMELVSV  VLGFAVPFSI  IAVFYFLL..  ...ARAISAS
  Consensus  -FP-ES----  W-TG--I--F  -LGF-VP--I  I--CY-L---  ---------G

            301                                                     350
somatostatin SSKRKKSEKK  VTRMVSIVVA  VFIFCWLPFY  IFNVSSVSVA  I.......SP
   d-opioid  SKEKDRSLRR  ITRMVLVVVG  AFVVCWAPIH  IFVIVWTLVD  IN......RR
         VIP  SDQEKHSSRK  I...IFSYVV  VFLVCWLPYH  VAVLLDIFSI  LHYIPFTCRL
  Consensus  S---K-S-RK  ITRMV--VV-  VF-VCWLP-H  IFV-----V-  I-------R-

            351                                                     400
somatostatin TPALKGMFDF  VVILTYANTC  ANPILYAFLS  DNFKKSFQNV  LCLVKVSGTE
   d-opioid  DPLVVAALHL  CIALGYANSS  LNPVLYAFLD  ENFKRCFRQ.  LCRTPCGRQE
         VIP  EHALFTALHV  TQCLSLVHCC  VNPVLYSFIN  RNYRYELMKA  FIFKYSAKTG
  Consensus  -PAL--ALH-  ---L-YAN-C  -NPVLYAFL-  -NFK--F---  LC------TE

            401                                                     450
somatostatin DGERSDSKQD  KSRLNETTET  QRTLLNGDLQ  TSI*TTQERN  MHTH*PSPDS
   d-opioid  PGSLRRPRQA  TTRERVTACT  PSDGPGGGAA  A*PTRPSP*T  PLPSEVIQRP
         VIP  LTKLIDASRV  SETEYSALEQ  NAK.......  ..........  ..........
  Consensus  -G-L-D----  --RE--T-ET  ------G---  ----------  ----------
```

APPENDIX III

pT7Blue T-Vector Kit, R & D Systems

A vector specifically constructed for cloning of PCR products. Taq DNA polymerase leaves single 3' A-nucleotide overhangs on the reaction products (Clark *et al* 1988). Therefore this vector contains compatible single T-nucleotide overhangs allowing direct ligation of the PCR product, but preventing self-ligation.

The vector comes ready prepared for ligation, by digestion with EcoR1 followed by the addition of single 3' dT residues at each end. A positive control ligation fragment of 50bp with single 3' dA end residues is provided.

pcDNA3 Mammalian Expression Vector, Invitrogen

A 5.4kb vector designed for high-level stable and transient expression in eukaryotic hosts. It contains the cytomegalovirus (CMV) promoter which gives high-level expression in a wide range of mammalian cells; a multiple cloning site polylinker; the bovine growth hormone (BGH) polyadenylation signal for polyadenylation of transcribed mRNAs; the neomycin resistance marker for the selection of stable transformants in the presence of G418. The vector also contains an SV40 origin and therefore will only replicate episomally in cell lines which express the SV40 large T antigen.

The positive control for transfection and expression is pcDNA3 with the 800bp chloramphenicol acetyl transferase (CAT) gene cloned into the HindIII site of the polylinker.

APPENDIX IV

Codes for nucleotide sequence degeneracy (chapter 3)

M = A or C
K = G or T
N = A, C, G or T
R = A or G
Y = C or T
W =A or T
S = G or C
D = A, G or T
H = A, C or T

1kb Marker (Gibco BRL) (chapters 3 & 4)

a	12 000 bp	k	2 000 bp
b	11 000 bp	l	1 600 bp
c	10 000 bp	m	1 000 bp
d	9 000 bp	n	500 bp
e	8 000 bp	o	400 bp
f	7 000 bp	p	350 bp
g	6 000 bp	q	300 bp
h	5 000 bp	r	200 bp
i	4 000 bp	s	100 bp
j	3 000 bp		

Calculation of Standard Error of the Mean (chapter 5)

$$\text{Standard Deviation} = \Sigma \frac{(x'-X)^2}{n-1}$$

$$\text{Standard Error of the Mean (S.E.M.)} = \frac{\text{S.D.}}{n}$$

where
n = number of saturation assays
x'= individual value
x = mean value

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